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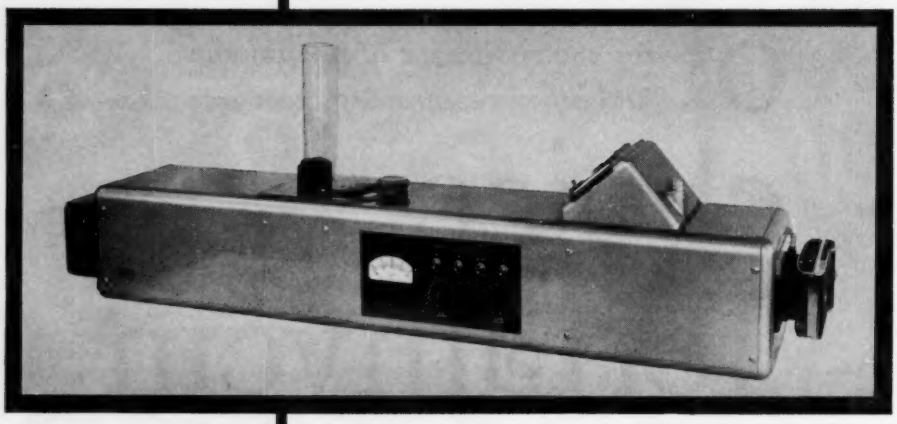

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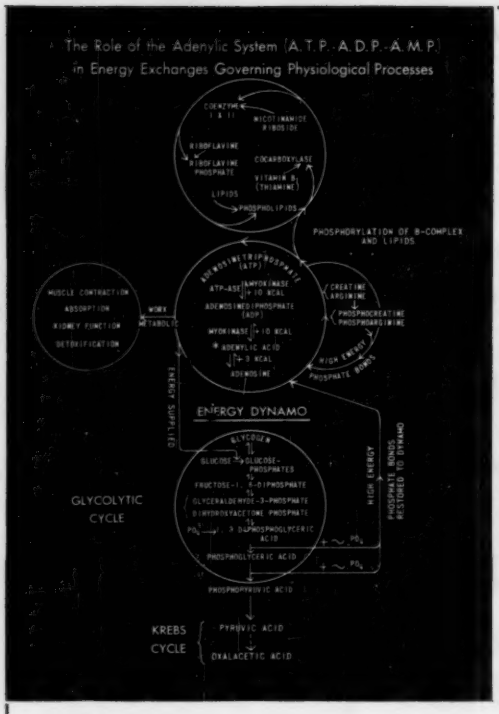

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THE RELATION OF NEUTRAL FAT TO LACTESCENCE OF SERUM [1]

By MARGARET J. ALBRINK, EVELYN B. MAN, AND JOHN P. PETERS

(From the Department of Internal Medicine, Yale University School of Medicine, New Haven, Conn.)

(Submitted for publication June 30, 1954; accepted October 6, 1954)

It has long been recognized that the cloudy or lactescent serum occurring in certain diseases contains an abnormally large amount of neutral fat (1), though a quantitative relationship between neutral fat and lactescence has not been established. In the present investigation the ultracentrifuge has been utilized to study the suspended lipid particles which are responsible for such lactescence. By analyzing a variety of clear and lactescent sera having high concentrations of one or more lipid components the relationship of neutral fat to lactescence has been verified and quantitatively defined.

METHODS

Serum lipids were determined before and after the removal of the suspended lipid particles by flotation in the ultracentrifuge. The technique of Van Eck, Peters, and Man (2) was modified as described below to permit the more complete separation of the lipids present as visible particles from the lipids present in clear solution. The particulate lipids which caused turbidity and were removed by centrifugation shall be referred to as "insoluble" lipid, while the lipids present in the clear subnatant after centrifugation shall be designated "soluble" lipid. The visual appearance of the serum was thus the criterion used in determining the state of solubility. No effort was made to classify the differential flotation characteristics of the lipids, the ultracentrifuge being used simply to remove the particles causing turbidity. Although no measurement of particle size was made, it is assumed that the insoluble lipids ranged from 0.1 μ in diameter, the size just sufficient to scatter light (3), to 1 μ, the size of the larger chylomicrons.

Duplicate 5 ml. portions of undiluted serum, in no instance more than 24 hours old, were measured accurately into two 13.5 ml. lustroid tubes. The serum was spun for one hour at 18,000 rpm (about 20,000 g.) at room temperature in a Spinco Model L Preparative Ultracentrifuge. After centrifugation the insoluble matter was visible on top as a buttery yellow layer in very lactescent serum, and as a faint white film in clear or slightly lactescent serum. The clear subnatant serum was removed from the bottom of each tube by piercing the "cream" layer with a No. 17, 2-½ inch needle, and inserting through this a long No. 20 needle to which was attached a Van Slyke pipette with a Luer-adapted tip. An exact quantity of the subnatant fluid was withdrawn into the pipette, leaving the "cream" layer floating undisturbed on top of the remaining serum. As the subnatant fluid was removed, exquisite care was taken to keep the tip of the outer needle just below the "cream" layer. In this way the fatty material which adhered to the outer needle was excluded from the clear subnatant fluid.

When the serum was moderately or grossly lactescent, the original serum and the clear subnatant fluid obtained after centrifugation were analyzed for lipids. The insoluble lipids were calculated by subtracting the lipids of the clear subnatant fluid from those of the original serum.

When the serum was clear or slightly lactescent and the anticipated difference between the original serum and the subnatant fluid was small, exactly 3 ml. of the subnatant fluid were removed from the bottom of the tube, and the "cream" layer was resuspended in the remaining 2 ml. of serum by gentle agitation of the lustroid tube. Any lipid adhering to the wall was scraped off and resuspended in the solution using the No. 17 needle as a stirring implement. This suspension was flushed in and out of the pipette-needle apparatus to insure complete mixing of the insoluble fat with the remaining subnatant fluid. In this way all the particulate lipid originally present in 5 ml. was concentrated in 2 ml. By subtracting the lipid values of the subnatant fluid from those of the "cream" suspension and multiplying the difference by two-fifths, the concentration of the insoluble lipids in the original serum was calculated. In the interests of uniformity all results were expressed as concentrations in the original serum (equal to the sum of the soluble and insoluble lipid) and in the clear subnatant, the difference between the two being the insoluble lipid. Although the differences appear small in the case of clear sera, they have added reliability because they are derived from the differences actually measured, which were over two times as great.

The accuracy of this method depends on the uniformity of distribution of lipids in the clear subnatant serum. Van Eck, Peters, and Man (2) found no consistent variation of lipids or protein of clear sera at different levels of the centrifuge tube. These findings have been essentially confirmed, with the exception that the

[1] Aided in part by a Grant from the United States Public Health Service and by an Institutional Grant from the American Cancer Society.

protein was from 0.1 to 0.3 gm. per cent higher in the subnatant than in the original serum. This layering did not affect the lipids, which were the same at different levels of the subnatant fluid.

In heavily lactescent sera, the separation of the insoluble lipids was not quite complete, since there was a zone of diminishing lactescence extending about one-third or one-half of the way from the top buttery layer into the tube. Thus in one lactescent serum in which the lipids were determined in the top buttery disc and in three equal layers of the subnatant from top to bottom, the concentration of neutral fat in the original serum was calculated to be 85.6 mEq. per L. Assuming the bottom clear layer, in which the concentration of neutral fat was 19.5 mEq. per L., to be free of insoluble lipid, the insoluble neutral fat calculated by difference was 66.1 mEq. per L. Of this, 51.7 mEq. were in the top buttery disc, 10.3 mEq. in the layer immediately under the disc and 4.1 mEq. in the middle layer. Cholesterol and phospholipids were similarly concentrated in the top. Therefore, in moderately or greatly lactescent sera the subnatant sample was taken from the bottom clear zone, and the insoluble lipids calculated as the difference between the original serum and the bottom-most layer. Any small error introduced by the incompleteness of the separation was thus minimized.

The lipids, which were determined by methods previously described (4), included total titratable fatty acids after hydrolysis of the lipids, total cholesterol and in many cases free cholesterol, and lipid phosphorus. Neutral fat fatty acids were calculated indirectly.[2]

[2] Neutral fat fatty acids were calculated from the following formula (4):

Neutral fat fatty acids in mEq. per liter = total fatty acids − (cholesterol fatty acids + phospholipid fatty acids), where cholesterol fatty acids in mEq. per liter =

$$\frac{10 \times \text{esterified cholesterol (mg. \%)}}{386}$$

and phospholipid fatty acids in mEq. per liter =

$$\frac{10 \times [(0.80 \times 2) + 0.2] \text{ lipid phosphorus (mg. \%)}}{31}$$

Esterified cholesterol was calculated by subtracting the free cholesterol from the total cholesterol. When free cholesterol was not determined, a ratio of free to total cholesterol of 0.28, the average ratio of normal sera (4), was assumed in order to estimate the ester. In these instances the spaces under "free cholesterol" and "per cent free cholesterol" in the tables were left blank.

The estimation of neutral fat is subject to the combined errors of the other determinations. It is also based on the assumption that 20 per cent of the total phospholipids carry one fatty acid molecule, and 80 per cent carry two. Deviations from this distribution would contribute further error, but it is unlikely that such deviations are great enough to be of serious consequence. Another assumption which requires verification is that all fatty acids not accounted for as phospholipid or cholesterol ester are present as triglyceride (neutral fat). The possibility that a portion of the calculated "neutral fat" is present as unesterified or free fatty acids, the presence of which has been reported after heparin administration (5–7), remains as another source of error, but preliminary studies have revealed that free fatty acids do not occur to an appreciable extent in normal serum, in post-prandial lipemia, in lactescent serum, or in chyle.

Cholesterol and lipid phosphorus are expressed in mg. per cent while total fatty acids and neutral fat are expressed in mEq. per liter, the terms in which they are actually measured.

In two instances the insoluble lipid was washed free of the subnatant serum by three-fold centrifugation with saline, and the lipid material so obtained was analyzed for lipids and proteins. Proteins were determined by the Kjeldahl method.

RESULTS

The sera fall into two rather clear-cut groups, designated "clear" and "lactescent." This division was to some extent arbitrary, many of the "clear" group showing the faint opalescence common in normal serum. The category "lactescent" was reserved for those sera having a definite cloudiness. The lipid values for the clear sera are given in Table I and those for the lactescent sera in Table II, with the exception of those diabetics whose serum changed from lactescent to clear while they were under observation. These are listed in Table III to show the chemical changes coincident with recovery from acidosis and clearing of serum.

The clear sera were found in patients with diabetes, liver diseases, obstructive jaundice, xanthomatosis of the hypercholesterolemic type, and hypothyroidism. The chemical findings, shown in Table I, were notable in that the neutral fat was in many instances within the normal range of 0 to 6 mEq. per liter (4) and never greater than 15.2 mEq. per liter. The cholesterols and with one exception the phospholipids were elevated, according to standards established in this laboratory (4).

In keeping with the clear appearance of these sera was the almost negligible quantity of insoluble lipid, most of which was neutral fat. Two and one-half mEq. of insoluble neutral fat fatty acids per liter was the greatest concentration that was present without distinctly clouding the serum.

The lipid values of the lactescent sera are given in Tables II and III. The conditions represented in this group were essential hyperlipemia, acute pancreatitis, hyperlipemia secondary to nephrosis

or diabetic acidosis, and in one instance (No. 12) chronic alcoholism, the lactescence being noted after an alcoholic bout, and disappearing when alcohol was withdrawn.

While all lipid elements were elevated in the lactescent sera, the neutral fat differentiated this group most strikingly from the group with clear sera. When the neutral fat of the original serum was below 14 mEq. per liter, the serum was clear, with the exception of that of No. 22 (Table III), which was slightly lactescent when the neutral fat was only 12.4 mEq. per liter. Lactescence appeared variably above 14 mEq. per liter, and definitely above 20 mEq. per liter of neutral fat. When the neutral fat was 90 mEq. per liter or higher, the normal straw color of the serum was completely obscured by an opaque milky appearance. The insoluble material causing the lactescence was composed chiefly of neutral fat, but it also included considerable quantities of cholesterol and phospholipid. As neutral fat rose, increasingly larger proportions of cholesterol and phospholipid were found in the insoluble fraction, though there was no constant relationship between the concentration of either of these and lactescence. The soluble cholesterols in the markedly lactescent sera (neutral fat greater than 90 mEq. per liter) were between 90 and 321 mg. per cent, though the total cholesterols of these sera were between 526 and 1508 mg. per cent. In clear sera the soluble cholesterol was as high as 831 mg. per cent.

The free cholesterol was in the normal range

TABLE I

Serum lipids before and after centrifugation: Patients with elevated cholesterol, clear serum

Patient	Unit number Age Sex	Date		Total fatty acids	Cholesterol			Lipid phosphorus	Neutral fat fatty acid	Comment
					Total	Free	Free / Total			
				mEq./L.	*mg. %*	*mg. %*	*%*	*mg. %*	*mEq./L.*	
1	A67585	3/13/53	Original	21.7	400	167	42	20.0	4.0	Biliary cirrhosis.
	36 F		Subnatant	20.3	386	157	41	19.2	2.6	Serum icteric
			Difference	1.4	14	10	71	0.8	1.4	
2	C21335	4/13/53	Original	24.2	423	116	27	16.2	6.8	Hypopituitary with
	53 F		Subnatant	20.9	378					hypothyroidism.
			Difference	3.3	45					Faintly lactescent
3	C11339	2/25/53	Original	26.9	559	162	29	19.7	5.6	Hypercholesterolemic
	29 M		Subnatant	24.6	535	154	29	19.3	3.6	xanthomatosis
			Difference	2.2	24	8	33	0.4	2.0	
4	Ros.	6/8/53	Original	22.2	402	116	29	12.0	7.8	Hypercholesterolemic
	40 M		Subnatant	20.6	391			10.5	7.4	xanthomatosis
			Difference	1.6	11			1.5	0.4	
		10/7/53	Original	22.8	362	106	29	15.9	7.0	
			Subnatant	21.6	317			15.8	6.6	
			Difference	1.2	45			0.1	0.4	
5	Ave.	4/21/53	Original	18.8	394	106	27	12.9	3.9	Hypercholesterolemic
	33 M		Subnatant	17.9	368	98	27	12.4	3.2	xanthomatosis
			Difference	0.9	26	8	31	0.5	0.7	
6	A66992	1/5/53	Original	35.1	412	121	29	19.0	15.2	Diabetic ketosis.
	48 F		Subnatant	30.5	389	116	30	18.4	12.7	Faintly lactescent
			Difference	4.6	23	5	22	0.6	2.5	
7	40–11–26	1/6/54	Original	45.6	762	702	92	57.0	11.2	Biliary cirrhosis.
	42 F		Subnatant	42.6	722			52.0	11.3	Serum icteric
			Difference	3.0	40			5.0	−0.1	
		1/13/54	Original	48.3	831	746	90	68.1	7.2	
			Subnatant	44.5	851					
			Difference	3.8	−20					
8	39–92–70	12/2/53	Original	40.8	555	519	93	42.8	14.1	Carcinoma of the bile
	66 F		Subnatant	39.4	545			41.0	14.3	duct with obstructive jaundice. Serum icteric
			Difference	1.4	10			1.8	−0.2	

TABLE II

Serum lipids before and after centrifugation: Patients with lactescent serum

Patient	Unit number Age Sex	Date		Total fatty acids	Cholesterol Total	Cholesterol Free	Cholesterol Free/Total	Lipid phosphorus	Neutral fat fatty acid	Comment
				mEq./L.	*mg. %*	*mg. %*	*%*	*mg. %*	*mEq./L.*	
9	Som. 50 M	10/30/52	Original	56.2	444					Diabetic acidosis
			Subnatant	41.8	416					
			Difference	14.4	28					
10	16572 57 F	3/31/53	Original	234.6	671	275	41	31.8	206.5	Diabetic ketosis with xanthomatosis
			Subnatant	29.7	127	54	42	10.9	21.6	
			Difference	204.9	544	221	41	20.9	184.9	
										Nephrosis
11	39-38-38 3 M	8/21/53	Original	187.0	1045	271	26	30.0	149.6	Serum total proteins, 3.30 gm. %
			Subnatant	44.4	280			17.0	26.7	Albumin, 0.8 gm. %
			Difference	142.6	765			13.0	122.9	Globulin, 2.5 gm. %
12	Yan. 45 M	8/20/53	Original	64.2	457	286	62	32.0	45.7	Alcoholic fatty liver
			Subnatant	31.8	263	168	63	11.0	21.8	
			Difference	32.4	194	118	61	21.0	23.9	
13	Brys. 40 M	11/11/53	Original	226.7	857	380	44	32.2	195.6	Essential hyperlipemia with xanthomatosis
			Subnatant	29.3	157	58	37	9.7	21.6	
			Difference	197.4	700	322	46	22.5	174.0	
14	B60586 31 F	11/17/53	Original	51.3	331	100	30	19.0	34.3	Diabetic acidosis
			Subnatant	30.5	217			14.9	17.9	
			Difference	20.8	114			4.1	16.4	
15	B78936 50 F	2/11/53	Original	135.4	526	195	37	24.2	113.4	Essential hyperlipemia
			Subnatant	17.6	90	46	51	11.3	10.0	
			Difference	117.8	436	149	34	12.9	103.4	
		3/26/53	Original	199.1	808	341	42	31.0	169.0	
			Subnatant	35.1	187	74	40	12.0	25.2	
			Difference	164.0	621	267	43	19.0	143.8	
		3/30/53	Original	185.5	940	325	35	35.0	149.6	
			Subnatant	31.8	179					
			Difference	153.7	761					
		9/14/53	Original	140.5	717			23.4	114.9	On 7/9/53
			Subnatant	26.4	160			11.5	17.3	Serum total proteins, 6.32 gm. %
			Difference	114.1	557			11.9	97.6	Albumin, 3.49 gm. %
										Globulin, 2.83 gm. %
		1/18/54	Original	133.0	705	176	25	27.0	103.0	
			Subnatant	27.5	202			12.3	16.4	
			Difference	105.5	503			14.7	86.6	
16	C63179 35 F	12/29/53	Original	46.6	385					Diabetic acidosis not fasting
			Subnatant	31.3	294					
			Difference	15.3	91					
17	84107 55 F	2/22/54	Original	47.7	456	149	33	20.0	28.1	Kimmelstiel Wilson's Disease
			Subnatant	34.2	424			16.9	16.9	
			Difference	13.5	32			3.1	11.2	Serum total proteins, 3.85 gm. %
										Albumin, 1.12 gm. %
										Globulin, 2.73 gm. %
18	40-53-29 2 M	4/1/54	Original	31.0	279	88	32	16.0	16.8	Possible nephritis
			Subnatant	22.0	282			12.0	10.0	Serum total proteins, 8.1 gm. %
			Difference	9.0	−3			4.0	6.8	Albumin, 5.0 gm. %
										Globulin, 3.0 gm. %
19	Ep. 21 F	4/21/53	Original	37.9	315	90	29	13.8	23.1	Acute pancreatitis
			Subnatant	30.7	267	77	29	11.8	18.9	
			Difference	7.2	48	13	27	2.0	4.2	

TABLE III

Serum lipids before and after centrifugation: Patients with lipemia of diabetic acidosis who showed clearing of serum with treatment

Patient	Unit number Age Sex	Date		Total fatty acids	Cholesterol Total	Cholesterol Free	Cholesterol Free/Total	Lipid phosphorus	Neutral fat fatty acid	Comment
				mEq./L.	*mg. %*	*mg. %*	*%*	*mg. %*	*mEq./L.*	
20	37-94-70 12 F	11/4/52	Original	126.0	636	256	40	33.5	96.8	Diabetic acidosis.
			Subnatant	23.8	122	60	49	10.0	16.4	Very lactescent
			Difference	102.2	514	196	38	23.5	80.4	
		11/10/52	Original	23.7	337	104	31	14.2	9.3	After recovery, clear
			Subnatant	21.7	291			11.9	9.3	
			Difference	2.0	46			2.3	0	
21	B72326 30 F	5/12/53	Original	34.4	282					Diabetic acidosis.
			Subnatant	21.0	187					Moderately lactescent
			Difference	13.4	95					
		5/14/53	Original	19.2	217	62	28	9.6	9.7	After recovery, clear
			Subnatant	16.5	219			9.2	7.1	
			Difference	2.7	−2			0.4	2.6	
22	B82609 16 M	8/17/53 9 PM	Original	526.0	1219	581	48	53.0	478.0	Diabetic acidosis
			Subnatant	33.0	115			11.0	26.0	xanthomatosis
			Difference	493.0	1104			42.0	452.0	Very lactescent Not fasting
		8/18/53 2:40 PM	Original	401.0	1508	683	45	50.0	361.0	Very lactescent
			Subnatant	30.9	200	95	48	11.2	21.7	Not fasting
			Difference	370.1	1308	588	45	38.8	339.3	
		4:40 PM	Original	327.9	1433	633	44	41.7	284.0	Very lactescent
			Subnatant	33.0	187			10.8	24.1	Not fasting
			Difference	294.9	1246			30.9	259.9	
		8/19/53	Original	231.7	1370	575	42	41.0	187.8	Very lactescent
			Subnatant	44.6	321			16.4	30.4	Fasting
			Difference	187.1	1049			24.6	157.4	
		8/21/53	Original	105.0	1194	343	29	34.0	63.3	Moderately lactescent
			Subnatant	37.3	493	187	38	17.9	17.9	
			Difference	67.7	701	156	22	16.1	45.4	
		8/25/53	Original	33.6	661	195	29	15.9	12.4	Slightly lactescent
			Subnatant	20.7	417			12.5	5.8	Xanthomata fading
			Difference	12.9	244			3.4	6.6	
		8/28/53	Original	26.6	516			15.9	7.9	Slightly opalescent
			Subnatant	23.9	426			15.7	6.7	
			Difference	2.7	90			0.2	1.2	
		8/31/53								Serum total proteins, 5.78 gm. % Albumin, 2.73 gm. % Globulin, 3.05 gm. %

of 24 to 32 per cent of the total (4) in all of the clear sera in which it was determined except those from patients having definite evidence of liver or biliary disease. In contrast to this, it was common to find 40 to 48 per cent of the cholesterol in the free form in both the soluble and insoluble fractions of the lactescent group, although the patients from whom these sera were obtained had little evidence of liver disease, the common cause for such elevated ratios.

Protein, total fatty acids, and cholesterol of the washed "cream" layer were determined in two of the sera (Table IV). The protein concentrations, 0.34 and 0.11 gm. per cent, were small enough to be attributable to the error of the method (0.3 gm. per cent). Furthermore, the protein was calculated from the total nitrogen, assuming all of the latter to be in the form of protein. If a correction is made for phospholipid nitrogen, estimated from the lipid phosphorus values in

TABLE IV

Protein and lipid content of washed cream layer

Patient	Date	Protein	Fatty acid	Cholesterol
		gm. %	*mEq./L.*	*mg. %*
10	3/31/53	0.34	211	435
15	2/11/53	0.11	140	477

Table II, the protein values become 0.24 gm. per cent and 0.05 gm. per cent, which in turn constitute 2.5 and 0.8 per cent of the weight of the fatty acids present in the "cream." [3]

DISCUSSION

The partition of lipids between the soluble and insoluble fractions at once reveals that the concentration of neutral fat in serum determines the presence and degree of lactescence. This is shown in Figure 1, where the soluble and insoluble neutral fat are plotted against the total neutral fat, the insoluble neutral fat serving as an estimate of the degree of lactescence.

The invariable appearance of lactescence when the neutral fat exceeds 20 mEq. per liter, suggests that this level represents a physicochemical limit to the concentration of neutral fat in the subnatant serum. This suggestion is strengthened by the constancy of the soluble neutral fat, which was usually present in concentrations close to 20 mEq. per liter and which varied only between the extremes of 10 and 30.4 mEq. per liter in all of the lactescent sera studied. All further increments of neutral fat, as much as 452 mEq. per liter, occurred in the insoluble fraction.

These findings prompt a further hypothesis, that the primary defect responsible for lactescence is not in the clearing mechanism but in the removal of soluble neutral fat from serum. It seems probable that neutral fat is delivered to serum chiefly in the insoluble form, and that its conversion to

[3] A molecular weight of 269 is assumed for the fatty acids.

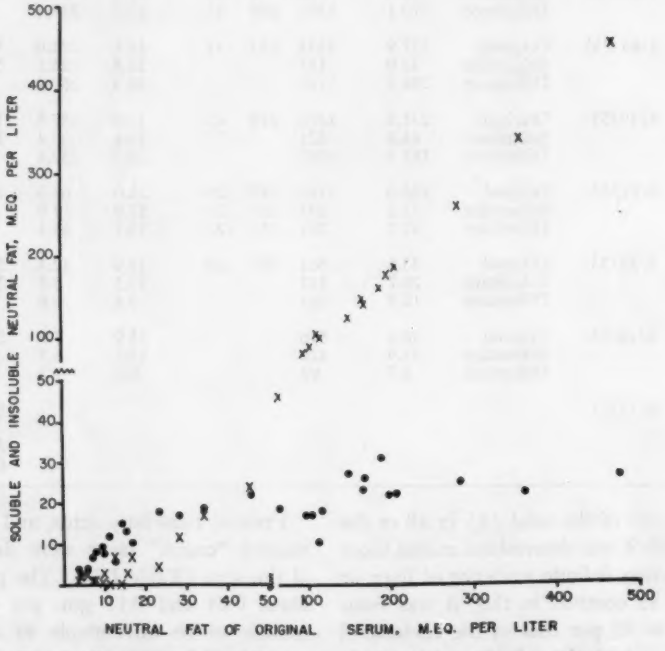

FIG. 1. RELATIONSHIP OF SOLUBLE AND INSOLUBLE NEUTRAL FAT TO TOTAL NEUTRAL FAT OF ORIGINAL SERUM

Soluble neutral fat ●. Insoluble neutral fat ×. Note the change in scale above 50 mEq. of neutral fat. Neutral fat is expressed as milliequivalents of its constituent fatty acids per liter.

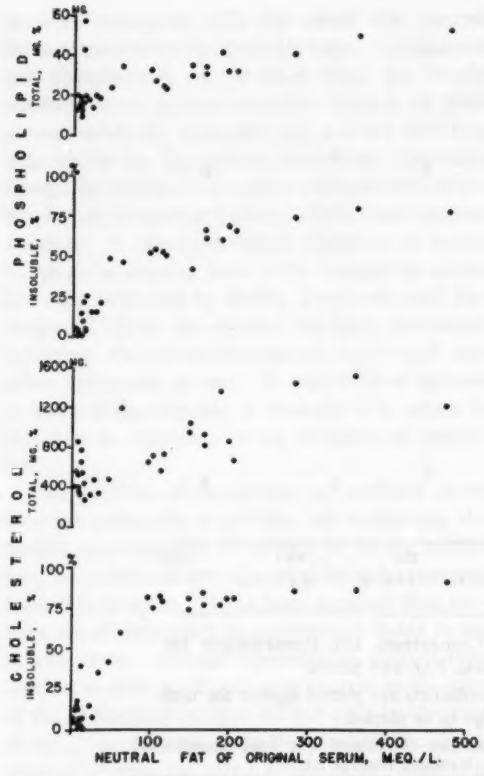

FIG. 2. THE RELATIONSHIP OF NEUTRAL FAT TO THE TOTAL AND THE INSOLUBLE CHOLESTEROL AND PHOSPHOLIPID PHOSPHORUS

Cholesterol is plotted both as the total amount in the original serum and as the per cent of the total which was insoluble and rose in the cream layer. Phospholipid phosphorus is charted similarly.

the soluble form is an early step in its metabolism. If there were a block in the removal of soluble neutral fat, the entry of insoluble neutral fat into the soluble fraction could progress only until the limitation noted above was exceeded, after which insoluble neutral fat, denied its normal route of egress, would accumulate, with resultant lactescence. Such a scheme fits well with the data shown in Figure 1.

The presence of cholesterol and phospholipid as well as neutral fat in the "cream" layer may indicate that these substances are dissolved in the neutral fat particles and thereby denied access to the soluble fraction. This would explain the low or normal values of cholesterol and phospholipid in the soluble form, despite their presence in large amounts in the "cream" of lactescent sera (Tables II and III). In Figure 2 both the total concentration of cholesterol and phospholipid, and the relative proportions of each which were in the insoluble fraction, are plotted against the total neutral fat. Not only did cholesterol and phospholipid increase as neutral fat increased, but the relative proportions of each which were found in the insoluble fraction also increased, forming an asymptotic curve when plotted against total neutral fat.

Such a curve could be explained if the insoluble neutral fat were considered the second phase of a two phase system, serum: neutral fat. Any raw cholesterol (or phospholipid) introduced into such a system would then distribute itself between the two phases according to its solubility in each phase. The ratio of the concentration of cholesterol (or phospholipid) in the neutral fat phase to its concentration in the serum phase would be a constant, the distribution coefficient, such as that described by Bischoff, Stauffer, Gray, and Katherman (8, 9) for steroid hormones. Such a two phase system, in which the serum phase was of a rather constant quantity in all sera while the size of the neutral fat phase increased with increasing lactescence, would account for the appearance of increasingly greater proportions of cholesterol and phospholipid in the insoluble fraction as neutral fat rose (Figure 2). This does not indicate that cholesterol and phospholipid have exceeded their solubility in serum, but merely that they are more soluble in neutral fat.

By assuming such a two phase system, the distribution coefficient for cholesterol between the neutral fat and the serum phase was calculated for each serum in the following way:

A. Concentration of cholesterol in neutral fat phase = concentration of insoluble cholesterol in mg. per 100 gm. insoluble neutral fat.[4]

B. Concentration of cholesterol in aqueous phase = concentration of soluble cholesterol in mg. per 100 ml. of serum.

$\frac{A}{B}$ = distribution coefficient between phases A and B.

[4] Milliequivalents of neutral fat were converted to grams by assuming a molecular weight of 269 for neutral fat fatty acid.

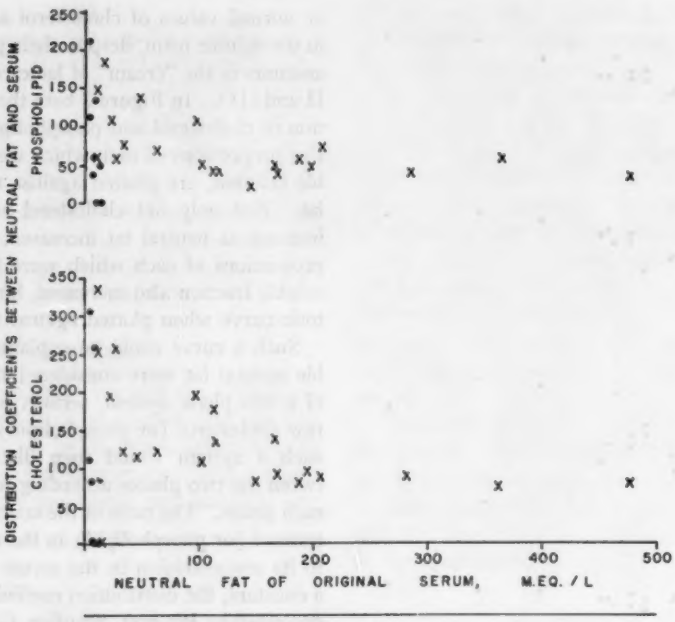

FIG. 3. DISTRIBUTION COEFFICIENTS OF CHOLESTEROL AND PHOSPHOLIPID BETWEEN INSOLUBLE NEUTRAL FAT AND SERUM

Clear serum ●. Lactescent ×. The coefficients are plotted against the total neutral fat. Two coefficients were too high to be plotted.

$$\text{Distribution coefficient} = \frac{\text{concentration of insoluble cholesterol (or lipid phosphorus) in mg. per 100 gm. insoluble neutral fat.}}{\text{concentration of soluble cholesterol (or lipid phosphorus) in mg. per 100 ml. clear serum.}}$$

Similar calculations were carried out for phospholipid. The coefficients for all the sera, both clear and lactescent, are plotted against the total neutral fat in Figure 3. There was great variability at low concentrations where the quantities of insoluble lipid were minute and therefore could not be measured with accuracy, but in those sera having a high neutral fat, the distribution coefficient for cholesterol between insoluble neutral fat and serum was quite constantly about 80, and that of phospholipid about 50. This suggests that the distribution of cholesterol and phospholipid between the soluble and insoluble fractions depends to some extent on their relative solubility in neutral fat and serum.

Such a hypothetical solubility system, which may account for all the observed lipid characteristics of lactescent serum, may be summarized as follows:

Failure to remove soluble neutral fat from serum → accumulation of first soluble, then insoluble neutral fat with resultant lactescence → distribution of cholesterol and phospholipid between serum and insoluble neutral fat on a basis of relative solubility.

It is recognized that such a scheme is an oversimplification, since the solubility of lipids *per se* in aqueous solution is extremely limited. The lipids entering into such true aqueous solution are probably taken up immediately by some substance, probably protein, in combination with which their solubility is greatly increased. The entry of any lipid into the serum fraction would depend both on its solubility in water, and on the avidity with which it is taken up by proteins. In lactescent serum it appears that the proteins cease to remove neutral fat from the aqueous phase, which in turn

becomes saturated, with the result that neutral fat accumulates in the insoluble form. Cholesterol and phospholipid, on the other hand, are largely withheld from aqueous solution because of their greater solubility in neutral fat, and are therefore unavailable for lipoprotein formation. Insoluble neutral fat therefore competes with proteins in removing cholesterol and phospholipid from aqueous solution. A physicochemical alteration of serum lipoproteins causing them to be trapped in serum has been proposed by Byers, Friedman, and Rosenman (10) to account for the high cholesterol following the administration of cholic acid and other detergents to rats. It would be of interest to know if the trapping of cholesterol in serum in this case is secondary to an elevation of neutral fat.

The problem of lactescence as outlined above becomes primarily a problem of explaining the limited concentration of neutral fat in the soluble fraction and the failure of neutral fat to be removed from this fraction. It has been assumed that proteins are all-important in maintaining lipids in the soluble state. Normal lipoproteins, though they contain as little as 25 per cent protein in the case of the β lipoproteins and 65 per cent in the case of the γ lipoproteins, share the physical characteristics of protein, including solubility in water (11, 12). Although the cholesterol and phospholipid content of these lipoproteins has been determined (11–14), little is known of their neutral fat content. Turner and his co-workers (15) presented ultracentrifugal evidence for one or more soluble lipoproteins containing neutral fat, and it is probably safe to conclude that neutral fat owes its solubility to its combination with protein.

In support of the importance of protein in maintaining lipids in the soluble state is the virtual absence of protein from the insoluble lipids of lactescent serum. The protein content of insoluble lipid obtained in the present study was less than 3 per cent by weight (Table IV). Van Eck, Peters, and Man (2) were able to wash the insoluble lipid of postprandial hyperlipemia free of protein. In the chemical method of fractionating lipoproteins (11), a yellow scum obtained after centrifugation is discarded, but the authors state that it probably contains free lipid. Lindgren, Elliott, and Gofman (16) state that the lipid material accumulating at the top of native serum spun for 24 hours at 104,000 g., which included by their classification the Sf 17 and higher particles, contained little or no protein, the quantities found being too small for accurate chemical analysis. Chylomicrons, which could be removed at 9500 g. in 10 minutes, contained nothing but neutral fat and one per cent cholesterol (16). The particles removed by the relatively mild centrifugation of the present study probably included only the larger particles of the Sf 40 class and greater, of minimal protein content. Particles of the Sf 10 to 20 group, which are said to scatter light (17), were probably not affected by the centrifugation, but their presence in the subnatant fraction in more than very small quantities is unlikely judging from the clarity of the "bottom" layer.

Kunkel and Slater (14), using a starch medium for the electrophoretic study of serum lipoproteins, demonstrated that the high neutral fat material of lipemic serum had a mobility similar to β lipoprotein. However, for technical reasons these authors were unable to measure neutral fat in the starch medium. Assuming that migration of neutral fat did occur, such a phenomenon could be explained as a loose affinity between globulin and free lipid particles, and does not offer proof that the lipids of such particles are in chemical union with protein. The evidence from the present and other studies favors the conclusion that the particulate lipids which cause lactescence are almost if not completely protein free and have the chemical and physical characteristics of free lipid rather than protein, while the lipids in solution are lipoproteins and owe their solubility to their combination with protein. The limited amount of protein available for lipoprotein formation may well be the critical factor imposing a limit to the concentration of neutral fat in the soluble fraction. Robinson and French (5) demonstrated that the amount of serum albumin present determined the degree of clearing that could be achieved by adding serum from a heparinized rat to chyle. Thoracic duct chyle which in many respects resembles lactescent serum (18, 19) also contains both soluble neutral fat and protein, but in concentrations about half as great as those in lactescent serum. Although the protein fractions of the lactescent sera were determined in only a few instances of the present study it is unlikely that albumin alone dictates the level of

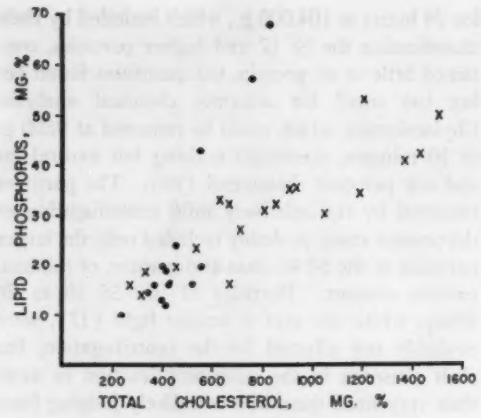

FIG. 4. RELATION OF LIPID PHOSPHORUS TO TOTAL CHOLESTEROL

Clear serum ●. Lactescent serum ×.

soluble neutral fat. The serum albumin of five patients (Nos. 11, 15, 17, 18, and 22) bore no evident relationship to the soluble neutral fat.

Since proteins are important in maintaining lipids in a soluble state, it may be that an abnormal protein-neutral fat complex in the soluble phase is responsible for the retention of soluble neutral fat in lactescent serum. Abnormal lipoproteins have been reported in a variety of pathological sera, but the specific relationship of these to lactescence is not known because neutral fat is rarely determined.

Repeated allusions appear in the literature suggesting that a disproportionately low phospholipid in relation to cholesterol and total lipid accounts for lactescence of serum, and conversely, a high phospholipid accounts for clarity of serum. This relationship was brought out by Ahrens and Kunkel (3) who compared the clear sera from patients with biliary cirrhosis and jaundice with the lactescent sera from patients with nephrosis, both groups having high total lipids. The high phospholipids in jaundiced patients are statistically related to the high free cholesterol also found in these patients. The relationship between phospholipid and free cholesterol is preserved regardless of whether the ratio of free to total cholesterol is normal (20, 21) or elevated (20). The increased ratio of phospholipid to total cholesterol in Ahrens' and Kunkel's (3) patients with biliary cirrhosis is probably merely an indication of the increased ratio of free to total cholesterol in these patients. If the ratios of free to total cholesterol were normal one would expect the phospholipid to total cholesterol ratios also to be normal and of no value in distinguishing between clear and lactescent sera. This was borne out in the present study where there were many sera with normal ratios of free to total cholesterol. The relationship of lipid phosphorus to total cholesterol was similar in both the clear and lactescent sera except for three points which showed a marked deviation above the rest, and represent the only sera with extreme elevations of the ratio of free to total cholesterol (Figure 4). In Figure 5 the relationship of lipid phosphorus to free cholesterol is the same in the clear as in the lactescent sera regardless of the cholesterol ratio. While phospholipids are undoubtedly important constituents of lipoprotein molecules it seems unlikely that a deficit of phospholipid is responsible for lactescence.

An elevation of neutral fat appears to be the only obligatory change necessary for the appearance of lactescence, and is sufficient to account for lactescence regardless of ratios among the other lipid fractions. Because of its limited solubility and its striking influence on the physical state of the other lipids, neutral fat deserves a place of respect in any study of the lipid-protein complexes by which lipids are transported in serum and across cell membranes.

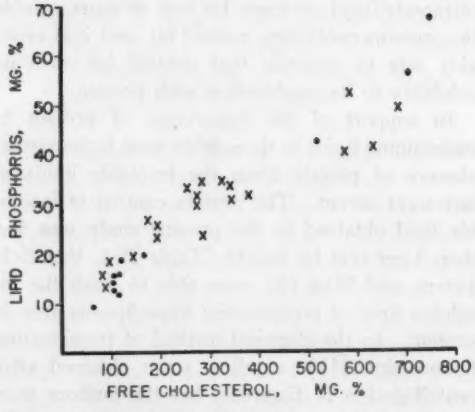

FIG. 5. RELATION OF LIPID PHOSPHORUS TO FREE CHOLESTEROL

Clear serum ●. Lactescent serum ×.

SUMMARY

1. The state of solubility of serum lipids was measured by comparing serum lipid values before and after removal of insoluble lipids by ultracentrifugation of a variety of clear and lactescent sera having cholesterol values greater than 300 mg. per cent.

2. Neutral fat was found to be the only lipid consistently associated with lactescence. When the neutral fat increased above 20 mEq. per liter the serum was invariably lactescent.

3. The soluble neutral fat of lactescent serum was found between the limits of 10 and 30.4 mEq. per liter despite variations of total neutral fat from 16.8 to 478 mEq. per liter. This was interpreted as indicative of a physiological limit to the concentration of neutral fat in the soluble form.

4. The solubility of phospholipids and cholesterol was decreased when neutral fat increased. The greater solubility of these substances in insoluble neutral fat than in serum is suggested as a possible reason for this relationship.

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AMMONIUM TOLERANCE IN LIVER DISEASE: OBSERVATIONS BASED ON CATHETERIZATION OF THE HEPATIC VEINS

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The concept that substances from intestinal contents, passing into the general circulation, may cause toxic symptoms has been of particular interest in the study of liver disease. In 1896, Nencki, Pawlow, and Zaleski (1) demonstrated that diversion of portal blood, containing large amounts of ammonium, into the general circulation by an Eck fistula resulted in an increase in ammonium content of peripheral blood. In cirrhosis of the liver and in patients with porta-caval anastomoses elevation of blood ammonium has frequently been found and in some cases correlated with neurological disturbances (2–10).

The reason for the elevation of blood ammonium in these cases has not been determined precisely. Van Caulaert, Deviller, and Halff (3, 10) suggested that in cirrhosis this abnormality of ammonium was related to the presence of portal collateral veins with shunting of portal blood directly into the systemic circulation. Kirk (2) supported this hypothesis with extensive studies.

To evaluate the roles of portal collateral circulation and of liver function in the control of blood ammonium levels, simultaneous sampling from antecubital and hepatic veins has been undertaken. To accentuate differences in ammonium content at these sites, and to test patients' ability to metabolize ammonium, a standard dose of ammonium chloride has been given by mouth. The results of such investigations, with corollary observations, form the basis of this report.

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A note on the measurement of ammonium in the blood

The measurement of blood ammonium is a flight after a Will-o'-th'-wisp (11), for it is generally agreed that ammonium is liberated as soon as blood is shed. Therefore, any single measurement made after drawing blood represents only a point on an ascending line of ammonium concentration.

By making timed serial determinations on the same sample of blood Conway and Cooke (12, 13) were able to construct a graph expressing the evolution of ammonium. From this graph, by extrapolation, they estimated the ammonium level at zero time, and concluded that there was no measurable ammonium in the circulating blood of normal individuals; they stressed that what was frequently measured as blood ammonium was in reality ammonium which had been liberated from other substances after the blood was drawn.

Conway and Cooke (13) found that blood drawn in an atmosphere of CO_2 did not liberate ammonium immediately, and suggested that CO_2 inhibited the breakdown of adenosine, which they had found to be the major source of the immediately liberated ammonium (14). Strehler, Haas, and Rupp (15) reported similar results and found that an atmosphere of CO_2 would decrease the ammonium content of blood drawn in air. This depression of ammonium liberation was presumed to act through an inhibition of an enzyme deaminating adenosine. Kalckar (16) found that CO_2 did not inhibit the deamination of adenosine by adenosine deaminase, nor did it catalyse reamination. In the present study no consistent significant inhibition of ammonium formation by CO_2 could be demonstrated. Other workers (6, 17) even though taking blood in an atmosphere of CO_2 have found high levels of ammonium in normal

blood, suggesting that CO_2 failed to inhibit ammonium liberation. We were also unable to suppress ammonium liberation by $AgNO_3$ which Zittle (18) found completely inhibited adenosine deaminase. Accordingly, in the results reported here blood was drawn in air, and no attempt to inhibit ammonium liberation was made.

In the present study the technique of serial ammonium determinations on the same blood sample has been used. Each determination was timed from the moment of beginning the collection of blood until an aliquot was pipetted into the diffusion chamber. Six analyses were made on each sample, and a graph was constructed expressing the changing concentration of ammonium. The extrapolated zero-time ammonium was determined, and the observations on 21 control subjects without hepatic disease (including 15 normals) supported Conway's conclusion that in the fasting subject without liver disease there is no measurable free ammonium in the circulating blood (Figure 1).

This method of performing ammonium determinations made it very obvious that substances other than ammonium were being measured. The nature of these compounds is not known, although adenosine may be one. The present data again demonstrated that, whatever the sources of

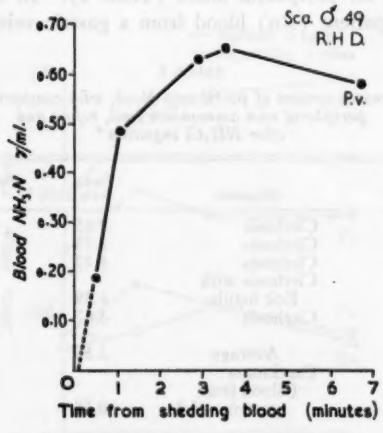

FIG. 1. DEMONSTRATION OF THE CHANGING AMMONIUM CONTENT OF A SINGLE BLOOD SPECIMEN, PLOTTED AGAINST TIME FROM SHEDDING

This chart is representative of others in patients without liver disease. RHD signifies Rheumatic Heart Disease.

what was measured as ammonium, the liberation was very rapid. Three minutes after the shedding of blood most of the ammonium was present as such, and repeated determinations after this time showed only a slow rise. Accordingly, for the purposes of the present paper the level of ammonium three minutes after shedding of blood has been taken as the ammonium content of the specimen. This leaves the question of zero-time ammonium, and rate of liberation to a day when more is known of the sources of blood ammonium and factors affecting its liberation.

It is emphasized that blood ammonia is an erroneous expression since at the pH of blood less than 1 per cent of ammonia is present in solution as such, the largest amount being in the ionized NH_4^+ form.

METHODS

Ammonium in blood was determined by a slight modification of Conway's microdiffusion technique (12). Scrupulously washed Conway units were prepared with the central absorbing chamber containing 0.7 ml. 1/4000 N HCl, with Tashiro's reagent as indicator. The outer ring contained 1.0 ml. saturated boiled K_2CO_3. Five-tenths ml. blood was added to the outer chamber from an accurately calibrated straight pipette. The timing of the blood samples was started with the first appearance of blood in the syringe, and ended when delivery from the pipette was completed. The lid, greased with paraffin-mineral oil mixture was applied, the unit rotated, and then put aside to incubate for 15 minutes at 15° C. Barium hydroxide, 0.00025 N, delivered from a horizontal pyrex, micrometer driven burette, was used to titrate excess acid. The $Ba(OH)_2$ was kept in a pyrex bottle stoppered with a soda lime trap to exclude CO_2. Using this technique titrations of blanks and standards were made with less than 2 per cent error.

After titration of bloods, and simultaneously run standards ($(NH_4)_2SO_4$ solution, 0.5 μg. ammonium nitrogen per ml.) and blanks (reboiled distilled water), the calculated ammonium content of the blood was corrected by a factor of 1.11, given by Conway (12) to correct for the difference in liberation of NH_3 by K_2CO_3 from blood and water. Results were then plotted against the recorded time intervals as μg. NH_4-nitrogen per ml.

A check was kept on two time intervals; the first the elapsed time between drawing the blood and pipetting into the units, and the second the fifteen-minute incubation period. The latter was chosen as convenient for our purposes, yet well below the time when ammonium may be liberated from glutamine.

As a check on NH_4Cl absorption, as well as to measure the most important end product of NH_4 metabolism, blood urea determinations were made by a sensitive

technique, accurate to ± 1 per cent (12). Blood glutamine measurements were made by the method of Boulanger and Osteux (19) accurate to ± 10 per cent.

For the ammonium tolerance test ammonium chloride, 3.0 gm. was given by mouth in gelatin capsules, and almost invariably provoked nausea, rarely vomiting.

Blood samples were taken simultaneously from an antecubital vein and through a catheter passed under fluoroscopic control into an hepatic vein. Specimens were drawn during the control period and 30, 60, and 120 minutes after NH_4Cl ingestion. Blood was collected in clean dry syringes without hemostasis. Boiled, NH_4 free potassium oxalate was used as the anticoagulant, as heparin was found to liberate NH_3 in the Conway unit.

Urine collections were made on 12 patients to measure NH_4 and urea output before and after NH_4Cl ingestion. The inability to obtain adequate urine flow, especially in cirrhotic patients, vitiated the significance of the measurements, and the effort was abandoned.

On nine occasions renal vein blood was obtained by catheterization. Blood was also taken from abdominal wall collateral veins in two patients with cirrhosis, and from various portal vein radicles in four patients at laparotomy, performed to explore the biliary system in three and for sub-total gastrectomy in one.

PATIENTS

The patients were divided by diagnosis into several groups.

I. No liver disease, 11 cases. These were hospital staff, 5, and patients suffering from chronic pulmonary or cardiac disease, who had normal liver function tests. They were studied at the time of cardiac catheterization for other purposes.

II. Acute virus hepatitis, 6 cases. This included both mild and severe cases. Two were again investigated during convalescence.

III. Cirrhosis, 26 patients. No distinction was made in this group between biliary cirrhosis (3 cases) and portal cirrhosis (23 cases). All of these patients had definite evidence of portal collateral vessels, demonstrated by contrast radiography, esophagoscopy, and/or portal venography (20). Three other patients who had cirrhosis without demonstrable portal collateral vessels were also studied.

IV. Miscellaneous, 8 cases. This diverse group included two with obstructive jaundice due to carcinoma, two with nephritis (one of whom was uremic), two with hepatosplenomegaly and normal liver function tests, one of whom had a normal liver biopsy. The other had abdominal tuberculosis. One patient presented the Chiari syndrome with invasion of the inferior vena cava and hepatic veins by renal carcinoma. One patient had a thrombosed portal vein following pylephlebitis, with esophageal varices and a normal liver by biopsy and function tests.

One patient with cirrhosis and two with normal liver function received no NH_4Cl, but had serial determinations of blood ammonium throughout the day while fasting to investigate diurnal variation.

The neurological signs and symptoms associated with liver disease have been extensively reviewed and are not described here. Of the patients investigated ten had such neurological changes associated with hepatitis or cirrhosis and are fully described elsewhere (9).

RESULTS

The mean "three-minute" fasting blood ammonium value in 21 control patients without liver disease was 0.79 μg. per ml., Standard Deviation 0.09.

In two patients with renal disease and normal liver function tests and one patient with cirrhosis and esophageal varices, one-half to two hourly blood samples were taken throughout the day while the patient fasted, and the fluctuation of ammonium levels was found not to exceed 0.1 μg. per ml. in antecubital or hepatic vein blood.

In seven resting control subjects simultaneous artery and vein blood samples were obtained from an extremity, and no significant difference in ammonium content at these two sites was observed. Other workers have reported similar results (21, 22).

In five patients the values for portal venous blood ammonium, obtained at laparotomy or from abdominal wall collaterals, averaged 2.7 times those for peripheral blood (Table I). In a further patient (Ru) blood from a gastric vein was

TABLE I

Ammonium content of portal vein blood, with comparison of peripheral vein ammonium level, before and after NH_4Cl ingestion *

Patient	Diagnosis	Portal vein	Peripheral vein
Br	Cirrhosis	2.65	1.12
To	Cirrhosis	2.75	1.04
Ne	Cirrhosis	4.27	1.06
Me	Cirrhosis with Eck fistula	4.39	2.53
Sw	Cirrhosis	3.67	0.89
	Average	3.55	1.33
Ru	Carcinoma (Blood from gastric vein)	0.58	
	After 3.0 gm. NH_4Cl by mouth		
To	Control	2.26	1.04
	60 minutes	6.48	2.75
Ne	Control	4.27	1.07
	60 minutes	6.83	1.52

* Ammonium expressed as μg. NH_4·Nitrogen per ml.

TABLE II

Ammonium content of renal vein blood, with comparison of peripheral vein ammonium level *

Patient	Diagnosis	Renal vein	Peripheral vein
Th	Tuberculosis	1.01	0.51
Me	Cirrhosis	1.21	0.79
Pe	Cirrhosis	1.35	0.99
Da	Cirrhosis	1.42	0.94
Me	Cirrhosis with Eck fistula	1.42	2.03
Ea	Cirrhosis	3.10	2.22
El	Cirrhosis	2.87	2.21
Sw	Cirrhosis	0.71	0.81
Au	Hepatitis	3.07	1.96
	Average	1.82	1.39

* Expressed as μg. NH_4·Nitrogen per ml.

found to have a low NH_4 content. NH_4Cl given by mouth to two patients with abdominal wall portal collaterals caused a rise in ammonium in peripheral vein blood, as well as in the portal collateral. In the latter the rise was to a higher absolute level, but in terms of per cent was not greatly different (Table I).

In nine patients (eight with liver disease, one with normal function tests) renal vein ammonium values averaged 30 per cent higher than peripheral vein (Table II). The height of the values was exaggerated because of the inclusion of patients with severe liver disease. Two patients with liver disease had values in the renal vein lower than in peripheral vein blood. In one of these the peripheral vein ammonium was abnormally high.

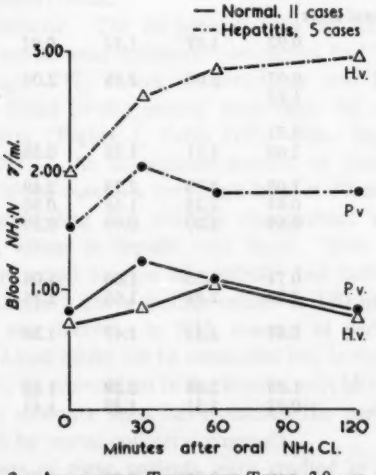

FIG. 2. AMMONIUM TOLERANCE TESTS, 3.0 gm. NH_4Cl BY MOUTH, COMPARING CONTROL PATIENTS (NORMALS) WITH PATIENTS WITH HEPATITIS

P.V.—peripheral vein H.V.—hepatic vein.

Ammonium tolerance tests

Controls. In eleven control subjects the mean value of ammonium in peripheral vein blood was slightly above that of the hepatic vein. After the ingestion of NH_4Cl the ammonium content of peripheral vein blood rose moderately with a rapid subsequent decline. The hepatic vein level rose more slowly, and continued to rise for a longer period. Both returned to control levels in 120 minutes. No control patient showed any abnormality of central nervous system function before or during the test.

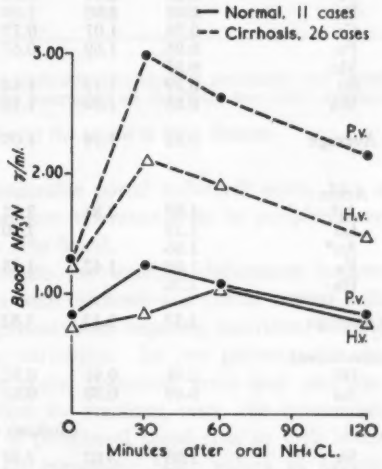

FIG. 3. AMMONIUM TOLERANCE TESTS, 3.0 gm. NH_4Cl BY MOUTH, COMPARING CONTROL PATIENTS (NORMALS) WITH PATIENTS WITH CIRRHOSIS AND PORTAL COLLATERAL CIRCULATION

P.V.—peripheral vein H.V.—hepatic vein.

Hepatitis. Four of the five patients studied during the acute phase of viral hepatitis showed elevated peripheral and hepatic vein ammonium levels in control specimens (Figure 2). In three of the four the hepatic vein ammonium content exceeded that of the antecubital vein blood. This became particularly marked after NH_4Cl ingestion, when the rise in ammonium values in hepatic vein blood far surpassed that of peripheral vein blood. Further, the return to control values was

delayed, and ammonium levels were above control at 120 minutes in both peripheral and hepatic vein blood. The one real exception to these findings was in a patient with mild, though acute hepatitis, whose only abnormal liver function test was an elevated bilirubin, but who had biopsy evidence of hepatitis. These data suggested that in patients with severe hepatitis ammonium

TABLE III

Ammonium tolerance tests in 51 patients. 3.0 gm. NH_4Cl by mouth †,‡

Patient	Peripheral vein				Hepatic vein			
	Zero	30	60	120 min.	Zero	30	60	120 min.
				Controls				
Sm	0.81	0.86	1.00	0.65	0.60	0.80	1.00	0.56
Wh	0.75	0.87	0.87	0.80				
Le	0.81	1.37	1.91	1.26	0.89	0.75	1.42	0.87
Hy	0.90	1.37	0.98	0.36				
Fe	0.86	0.84	1.09	0.90				
Pa	0.83	1.93	1.09	0.67				
Ce	0.78	1.07	0.79	0.77	0.87	0.52	0.75	0.76
Pu	0.95	1.69	0.67	0.79	0.72	1.12	0.63	0.67
Mc	0.65				0.62			
Su	0.89	1.37	1.44	0.88				
Wa	0.86	1.04	1.10	1.25	0.69	1.00	1.52	1.07
Average	0.82	1.24	1.09	0.83	0.73	0.84	1.06	0.78
				Hepatitis				
Acute								
Al*	1.02	2.84	2.81	2.45	1.33	4.43	5.36	4.48
Lo	1.25		1.40		1.25		2.55	
Au*	1.96				2.57			
Pa	1.03	1.42	1.25	1.19	0.87	0.81	0.65	1.48
Ha	2.36				3.80			
Average	1.52	2.13	1.82	1.82	1.96	2.62	2.85	2.98
Convalescent								
OF	0.91	0.91	0.91	1.13				
Au	0.49	0.88	0.85	0.18				
				Cirrhosis with portal collateral veins				
Sh	1.05	3.02	2.87	1.63	0.92	1.07	1.22	0.92
Li*	1.55	3.66	3.63	3.15				
Br	1.18	3.65	4.60	2.62	0.97	2.06	2.16	2.00
El*	2.22				1.75			
Li*	1.42	4.66	5.14	4.00				
Ea	2.21				3.31			
Si*	1.99	2.70	3.06	2.30	1.60	1.11	1.58	0.56
So	1.35	1.98	2.00	0.96				
We	0.73	1.77	1.28	0.98	1.08	2.28	2.23	2.49
Fe	0.68	2.62	1.60	0.80	0.64	2.25	1.48	0.90
Bre	0.77	2.02	1.58	1.28	0.89	1.00	0.99	0.50
To*	1.64	2.43	2.93	3.33				
To*	1.04		2.75					
Be*	1.39	5.51	4.90	3.24	0.71	2.55	1.60	1.06
We*	1.56	6.72	3.66	3.10	1.20	2.84	1.66	1.51
Ne	1.07		1.52					
Pe	0.99	1.79	2.19	1.69	0.87	1.12	1.47	1.38
Ha	1.33	3.46	2.98	3.02				
St	0.84	1.83						
Li	1.12	1.95	1.94	1.46	1.05	2.28	2.29	1.64
Sw	0.89	1.28	1.40	1.42	0.95	1.31	1.35	1.41
Da	0.94	2.39	2.26	2.16				
Me*	2.03	2.53	1.82	2.00				
Mu	1.02	3.54	3.86	2.41				
Mu	0.96	2.19	2.26	2.45	1.43	2.76	2.84	2.81
Ca	1.02	3.64	2.29	1.62	1.38	4.96	4.20	3.06
Average	1.27	2.97	2.63	2.17	1.25	2.12	1.93	1.48

TABLE III—*Continued*

Patient	Peripheral vein				Hepatic vein			
	Zero	30	60	120 min.	Zero	30	60	120 min.
				Miscellaneous				
Obstructive jaundice								
Pu	1.28	1.54	1.05	1.01	0.58	0.72	0.91	0.81
Cr	1.17	1.73	1.98	0.79	0.62	1.05	0.95	0.70
Nephritis								
Ca	0.93	1.00	0.86	0.81				
Ba	1.10	0.81	0.73	1.24				
(Uremia)								
Hepatosplenomegaly								
Sk	0.57		1.25		0.65		1.15	
Th	0.51	0.90	0.80	0.64	0.40	0.75	0.76	0.60
Chiari syndrome								
Ho*	1.52		3.50					
Thrombosis, portal vein; esophageal varices								
Ha	1.08	1.44	0.94	1.05	0.80	0.86	1.04	0.96

† Expressed as micrograms NH_4·Nitrogen per ml.

‡ All averages were first calculated excluding data where there were not simultaneous peripheral and hepatic vein samples. As this is a result almost identical to that obtained from averaging all the data, the latter averages have been given.

* These patients had neurological disturbances at some time during the course of their illnesses.

from the intestine passed through the damaged liver and so reached the systemic circulation.

One patient developed a tremor after the ingestion of NH_4Cl and this symptom persisted for three days.

Two patients in the convalescent stage of hepatitis, with normal liver function tests were found to have normal ammonium tolerance curves in peripheral blood.

Cirrhosis. The 26 patients who had cirrhosis and portal vein collateral circulation had higher average NH_4 values in peripheral and hepatic vein blood in the fasting state than did control subjects (Figure 3, Table III). After ingestion of NH_4Cl the ammonium content of peripheral vein blood rose to a very high level at 30 minutes, far exceeding the average concomitant rise in NH_4 values in hepatic vein blood. Both fell at a rate equal to that of controls, but both were significantly above fasting values at 120 minutes. The vast increase in NH_4 content of peripheral vein blood could not be accounted for, in these patients, by ammonium in the hepatic vein blood, and large amounts must have reached the peripheral blood by portal collateral channels.

Four of these patients were studied at a time when tremor and mental confusion were present; during the test their symptoms were exaggerated.

Three patients with cirrhosis who did not have demonstrable portal collateral veins, had normal ammonium tolerance tests in peripheral and hepatic vein blood.

Despite the striking differences between patients with cirrhosis and portal venous collaterals and patients with hepatitis, individual cases showed some variations. In one patient with cirrhosis, large portal collateral veins and excellent liver function by standard tests, the ammonium content of peripheral blood rose to high levels after NH_4Cl ingestion; NH_4 values in hepatic vein blood increased only slightly and were below the

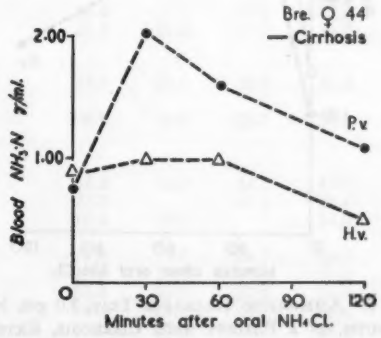

FIG. 4. AMMONIUM TOLERANCE TEST, 3.0 gm. NH_4Cl BY MOUTH, IN A SINGLE PATIENT WITH CIRRHOSIS, ESOPHAGEAL VARICES AND GOOD LIVER FUNCTION

P.V.—peripheral vein H.V.—hepatic vein.

fasting level at 120 minutes (Case Bre, Figure 4). This response demonstrated the importance of collateral channels in the production of abnormal ammonium tolerance in some patients with good liver function. Conversely, in an additional patient with cirrhosis, portal collateral veins and poor liver function (elevated bilirubin, abnormal thymol turbidity and low serum albumin), a marked increase in ammonium levels in blood followed the ingestion of NH_4Cl and was more marked in hepatic vein blood than in peripheral vein blood. Ammonium values at both sites were above the fasting at 120 minutes (Case Can, Figure 5). This case demonstrated that in cirrhosis with poor liver function NH_4 may pass through the damaged liver into the general circulation.

Miscellaneous. Two patients with obstructive jaundice due to carcinoma showed elevated peripheral vein ammonium levels in control, 30 and 60-minute specimens, but 120-minute values were below the fasting levels. The hepatic vein NH_4 values were within normal limits (Table III).

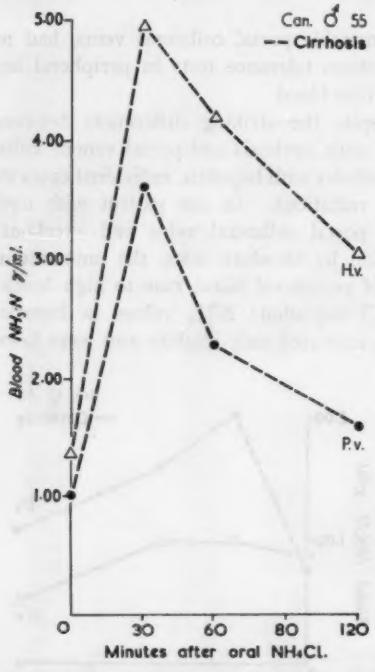

FIG. 5. AMMONIUM TOLERANCE TEST, 3.0 gm. NH_4Cl BY MOUTH, IN A PATIENT WITH CIRRHOSIS, EXTENSIVE PORTAL COLLATERAL CHANNELS AND POOR LIVER FUNCTION

Despite the high level of NH_4 he experienced no neurological disturbance during the test.

P.V.—peripheral vein H.V.—hepatic vein.

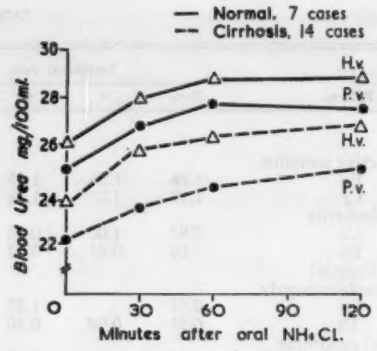

FIG. 6. BLOOD UREA DETERMINATIONS DURING THE AMMONIUM TOLERANCE TEST

Values in control patients compared with those in patients with cirrhosis.

P.V.—peripheral vein H.V.—hepatic vein.

One patient with nephritis showed a normal ammonium tolerance curve in peripheral vein blood. A second patient, with nephritis and uremia (blood urea 418 mg. per 100 ml.) had a fall in peripheral blood NH_4 after NH_4Cl ingestion, with a late rise (Table III).

The two patients with hepatosplenomegaly and normal liver function tests showed ammonium tolerance tests within normal limits in both peripheral and hepatic vein blood.

The patient with the Chiari syndrome had an elevated blood NH_4 in the fasting peripheral vein blood, and this rose higher 60 minutes after the ingestion of NH_4Cl.

The patient with normal liver, thrombosed portal vein and extensive esophageal varices secondary to pylephlebitis, had an elevated NH_4 response in peripheral vein blood after NH_4Cl. The hepatic vein figures were within normal limits (Table III).

None of the patients in this miscellaneous group had neurological symptoms at any time before or after NH_4Cl ingestion.

Blood glutamine studies

In seven control subjects there was no consistent or significant change in glutamine content of peripheral or hepatic vein blood after NH_4Cl

TABLE IV

Blood glutamine during ammonium tolerance tests, comparing levels in peripheral and hepatic vein blood *

Patient	Peripheral vein				Hepatic vein			
	Control	30	60	120 min.	Control	30	60	120 min.
				Controls				
Pa	13.0	13.3	12.6	12.2				
Fo	8.1	8.4	7.8	12.9				
Lo	7.4	11.0	10.9	13.6	9.4	8.7	10.0	9.6
Sm	14.5	12.0	15.4	12.0	10.2	11.5	12.4	12.0
Hy	12.5	13.4	15.4	12.5				
Wh	13.6	13.7	8.1	8.3				
Co	12.4	11.0	8.1	11.0	10.6	8.8	9.2	9.7
Mc	10.0				8.0			
Average	11.4	11.8	11.2	11.8	9.6	9.7	10.5	10.4
				Cirrhosis				
Ga	6.6		9.0	8.6	7.8	8.4	7.8	8.2
Sh	25.0	18.2	17.1	12.1				
Br	5.1	12.0	8.2	10.1	5.6	6.3	3.8	10.3
Mu	9.9	12.1	14.2	6.1				
Mu	9.8	13.2	11.6	13.9	12.7	8.4	9.8	12.4
So	13.4	11.4	9.8	7.8				
Si	13.2	11.5	9.8	10.2	11.1	11.9	10.1	13.6
Wo	14.2	12.6	11.8	12.6	7.7	5.7	10.2	17.9
Ca	10.6	13.6	14.4	15.0	11.5	12.4	12.4	12.9
Fe	7.3	17.1	12.0	12.7	10.2	15.1	15.1	19.6
Br	12.1	8.3	8.7	11.8	6.4	7.1	7.9	5.6
To	10.0		10.5					
Be	13.8	9.9		11.3	8.8	7.7	6.3	11.3
Li	7.1	6.7	8.5	12.2				
Pe	12.5	10.3	12.2	11.2	9.4	9.1	11.2	9.7
Average	11.4	12.1	11.3	11.1	9.1	9.2	9.5	12.2

* Expressed as mg. per 100 ml.

TABLE V

Blood urea during ammonium tolerance tests, comparing levels in peripheral and hepatic vein blood *

Patient	Peripheral vein				Hepatic vein			
	Control	30	60	120 min.	Control	30	60	120 min.
				Controls				
Pa	25.0	26.6	26.0	21.2				
Fo	18.1	19.7	21.7	22.5				
Lo	24.7	26.3	26.7	27.2	24.9	27.0	28.1	27.5
Sm	20.9	19.3	21.9	21.0	20.5	21.8	22.2	22.2
Hy	26.0	27.0	27.5	29.0				
Wh	29.0	33.0	35.0	36.0				
Co	31.6	35.0	35.2	35.6	33.0	34.9	35.9	36.4
Average	25.0	26.7	27.7	27.5	26.1	27.9	28.7	28.7
				Cirrhosis				
Ga	28.4	41.1	42.4	43.6	37.2	43.2	44.2	43.0
Sh	23.3	22.6	28.6	34.2	23.2		31.3	35.4
Br	21.6	21.0	21.0	20.0	18.4	23.6		22.0
Mu	20.0	20.9	23.4	24.1				
Mu	20.4	19.2	19.5	19.8	19.0	19.7	18.8	19.8
Si	30.7	31.2	31.7	32.1	29.8	29.9	31.0	31.0
Wo	21.6	23.2	23.4	23.1	23.0	22.5	21.1	25.6
Ca	25.3	26.2	26.1	27.0	26.8	27.2	26.9	27.6
Fe	14.8	15.2	15.8	16.2	15.2	16.6	16.3	17.1
Br	20.6	19.6	22.4	23.2	22.4	23.1	25.6	24.0
Be	25.1	25.6	25.3	26.2	27.0	28.2	27.8	28.6
Li	14.6	15.2	14.7	14.6				
We	18.5	20.6	19.1	18.8	18.6	21.0	18.8	19.6
Me	24.8	26.0	26.8	27.1	24.0	27.4	28.2	28.0
Average	22.1	23.4	24.3	25.0	23.7	25.7	26.4	26.8

* Expressed as mg. per 100 ml.

ingestion (Table IV). Similarly, in fourteen patients with cirrhosis there was no consistent response in blood glutamine to the administration of NH_4Cl by mouth. Individual cases varied widely, but averages showed no significant differences. Values below those of peripheral vein blood were obtained in two patients from renal vein blood.

Blood urea studies

Blood urea increased in seven control subjects and in fourteen patients with cirrhosis after NH_4Cl ingestion (Table V). Quantitatively the total rise was equal in the two groups, and the rate of rise was similar (Figure 6).

DISCUSSION

The data presented suggest two main sources of what is presently measured as blood ammonium in resting subjects: 1) The gastrointestinal tract; and 2) the kidneys. These results agree with previously published observations (1, 2, 21, 22).

The proof that a substance, or substances, from intestinal contents may provoke systemic disturbances must rest on three factors: 1) That the material must be absorbed in sufficient quantity from the intestine; 2) that it must then enter the general circulation in abnormal amounts; and 3) provoke toxic manifestations.

The data reported here confirm that NH_4Cl fulfills the first two of these requirements in patients with severe liver disease. The high fasting level of ammonium in portal blood and its rapid rise after an oral dose of NH_4Cl prove its rapid absorption (23).

The small increase in peripheral blood ammonium levels after NH_4Cl ingestion in control subjects is in marked contrast to results observed in patients with liver disease. In patients with severe acute hepatitis, or cirrhosis with poor liver function, the normal high gradient between ammonium content of portal and hepatic venous blood is diminished, and large amounts of ammonium reach the general circulation through the liver. In patients with good liver function, but with extensive portal collateral circulation, high peripheral blood ammonium levels may follow the ingestion of NH_4Cl. In these patients, however, the NH_4 gradient from portal to hepatic venous blood is normal, indicating that ammonium entered the peripheral circulation through some route other than hepatic veins, probably through the portal collateral channels. In patients with poor liver function and portal collateral veins both routes exist, and high levels of ammonium are found in peripheral blood. These results demonstrate the pathways taken by ammonium from intestine to peripheral blood, and confirm for NH_4Cl the second of the above factors.

Evidence was also obtained for the third factor, that NH_4Cl is, in fact, toxic. Five patients showed accentuation of neurological disturbances during the NH_4Cl tolerance test. Such symptoms were found only in those patients in whom the NH_4 levels remained elevated for a considerable period. Not all of the patients with prolonged high peripheral blood ammonium levels developed neurological symptoms, and such disturbances occurred in others when blood ammonium was only moderately elevated (9). There was no evidence of acidosis in any patient (9).

It is, at present, impossible to state that ammonium itself is *the* toxic substance, or to explain the mechanism of toxicity. It is certain that ammonium, or some material derived from it, can produce toxic changes in the central nervous system. Other nitrogenous substances may have a similar effect (6).

The rise in ammonium level of hepatic vein blood after an oral dose of NH_4Cl is a sensitive index of the capacity of the liver to metabolize ammonium, and therefore of hepatocellular function.

The present investigation cast no light on the metabolic abnormality in these patients. In the patients with portal collateral veins, the shunting of blood away from the normal site of ammonium metabolism could itself be responsible for high NH_4 levels in peripheral blood. In the presence of poor liver function, however, some abnormality in the metabolism of NH_4 in the liver is obvious. Little can be deduced of the role of glutamine synthesis in this mechanism from the present data. Glutamine is, however, quantitatively of minor importance as an end product of ammonium metabolism. Of far more importance is urea, the formation of which is primarily an hepatic function (24, 25). In these studies no difference between nor-

mal and cirrhotic subjects was found in the ability to synthesize urea.

Ammonium tolerance tests in the miscellaneous group of patients demonstrated an abnormal response in two patients with obstructive jaundice and one patient with the Chiari syndrome. No explanation is apparent for this abnormality in obstructive jaundice. The essentially normal response in the one patient with thrombosed portal vein, esophageal varices and good liver function must be regarded as unexplained, except insofar as all biological material varies in response.

CONCLUSIONS

1. In resting patients without liver disease there is no measurable ammonium in the circulating peripheral blood. Three minutes after shedding of blood appreciable amounts of ammonium have appeared, and this represents what is usually called blood ammonium.

2. Fifty-one ammonium tolerance tests have shown that patients with severe liver disease, following an oral dose of 3.0 gm. of ammonium chloride, exhibited an abnormally elevated ammonium content of peripheral vein blood.

3. Hepatic vein catheterization demonstrated that this abnormality was related to the failure of the liver to metabolize ammonium normally and/or to the presence of portal collateral veins, diverting high ammonium content portal blood around the liver.

4. Five of the patients with abnormal ammonium tolerance tests showed neurological symptoms during the test.

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THE INTRAVENOUS INFUSION OF THE STREPTOCOCCAL FIBRINOLYTIC PRINCIPLE (STREPTOKINASE) INTO PATIENTS[1]

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The streptococcal fibrinolytic principle (streptokinase) has been demonstrated to be an effective agent, *in vivo*, in patients as a means of mediating the relatively rapid liquefaction of extravascular fibrin coagulums (1). Streptokinase (referred to in this article as SK) has also been shown to be useful as a therapeutic reagent, by which massive extravascular clots may be eradicated by liquefaction and aspiration (2). These findings have received extensive confirmation. (See Footnote 12 to the list of References.)

It is the purpose of this article to describe the results of studies of the intravenous administration of SK into patients. The ultimate objective of the study is directed toward attempting to determine whether or not clots occurring within the vascular system as a result of disease may be subject to streptococcal fibrinolysis. It seemed desirable, however, to initiate the studies by making preliminary observations concerning the toxicity for patients of the currently available preparations of SK and the extent to which streptococcal fibrinolytic activity may be created within the general circulation.

In a previous report (3) intravascular clots artificially induced in the marginal ear veins of rabbits with sodium morrhuate proved to be susceptible to eradication by lysis when infusions of SK were introduced intravenously into the vein of the opposite ear. By the experimental procedure employed, the fibrinolytic system was rendered active within the circulating blood, and remained present for 6 to 12 hours depending on the amount of SK and the duration of the infusion. The intravascular clots liquefied within 3 to 7 hours of the beginning of the infusion.

[1] This study was supported by grants from the National Institute of Health, U. S. Department of Health, Education and Welfare, and Lederle Laboratory Division of the American Cyanamid Company.

In patients, the intravenous introduction of SK has proceeded with considerable caution. The amount of SK has been gradually increased beginning with doses as small as 50 units contained in 10 ml. of solution. In preparing solutions of SK for use in patients three variables have been taken into consideration: 1) Units of SK per injection, 2) concentration of SK in relation to total volume of solution; 3) duration of infusion.

As the amount of SK approached "therapeutic" levels, the most noteworthy toxic effects have been a pyrogenic response and depressions in blood pressure. Their occurrence and the use of drugs to counteract the untoward reactions will be subsequently described.

Of special importance has been the dosage of SK at which an effective streptococcal fibrinolytic system was created within the circulating blood of the patients.

The data contained in this article deal with the toxic effects and the measurements pertaining both to increments involved in intravascular streptococcal fibrinolysis, and also to alterations in complement and prothrombin time.

MATERIALS AND METHODS

Streptokinase. The SK was contained in Varidase®.[2] Varidase® is a concentrated and partially purified protein preparation containing other enzymes of streptococcal origin, notably streptodornase (SD), which was present in a ratio of 4 units of SK (3) to 1 unit of SD (4). Other enzymes that may be present are hyaluronidase and ribonuclease. It is estimated that active SK constitutes about 10 per cent of the protein in Varidase.® It is apparent, therefore, that even though partially purified, there is a considerable amount of extraneous material present in Varidase®. The amount of SK is estimated as approximately 100 units per *gamma* of nitrogen.

Up to the present time only two lots of the concentrate have been employed extensively, Lot 96A and Lot 333A.

[2] Supplied by Lederle Laboratories, Pearl River, N. Y.

Prepared in a dry form, each vial contained approximately 100,000 units of SK.

With each lot, the injections were initiated in very small amounts and low concentrations. The toxicities may be referable to extraneous and unknown substances present in undetermined amounts in different lots. Even though the activity of SK and the method of preparation of different lots is the same, it was not assumed that the toxicity of all lots would be comparable.

Preparation of infusions. Although different amounts of SK contained in different total volumes have been employed, in the observations reported in greatest detail in this article, 100,000 units of SK (one vial) was dissolved in 500 ml. of a saline-albumin solution to make a final concentration of 200 units of SK per ml. The diluent was prepared by adding purified, heated, sterile human albumin [3] to physiological saline in sufficient quantity to make a 1 per cent solution. Unpublished observations have demonstrated that the activity of SK in dilute solutions is preserved in the presence of 1 per cent albumin for at least 24 hours at room temperature.

Rates of infusion. Although varying rates of infusion have been employed, the main data were derived from introducing the 500 ml. of SK solution intravenously in 4 to 5 hours, or approximately 20,000 units of SK in 80 to 100 ml. per hour. Specific variation from this infusion rate will be considered in individual protocols.

Clinical observations. During the period of the infusion, pulse, temperature and blood pressure were taken at least every 15 minutes. The symptoms, if any, were also recorded. After termination of the infusion, the observations were generally continued for 24 to 48 hours.

Pre-infusion medication. The febrile response was counteracted with amidopyrine. In an effort to control the depression in blood pressure the antihistamine Chlor-trimeton® Maleate [4] was administered. The amidopyrine (0.6 gm.) and Chlor-trimeton® Maleate (4 mgm.) were given four times a day for two days preceding the infusion and continued until 24 to 36 hours after termination of the infusion. Their effects will be subsequently described.

That drugs belonging to the antihistamic group have a pressor effect has been reported by Yonkman, Chess, Mathieson, and Hansen (5) in studies of Pyribenzamine®.[5] Since Chlor-trimeton® has proved to exert the minimum of untoward side effects, it has been employed in this study.

Laboratory determinations. The tests fall into two general categories: a) Those primarily associated with the streptococcal fibrinolytic system and its activity; and b) collateral hematological tests and urine examinations.

SK activity tests. The essential test for determining *streptococcal fibrinolytic activity* was performed as follows:

The patient's blood was drawn in a syringe washed with saline (as for a Lee-White clotting time) and the fibrinolytic activity was determined on the undiluted whole blood *in vitro* by adding 2 ml. of fresh venous blood to a previously siliconed,[6] graduated, 10 to 15 ml. centrifuge tube (Figure 1). The rubber stopper was then inserted, with its attached wire,[7] and the tube placed in a waterbath at 37.5°C. The blood usually clotted in the tube within 15 minutes, and the time and amount of clot lysis were then noted at frequent intervals for 48 hours. Inasmuch as no differences were observed in the amount or time of lysis during the second 24-hour period, only the data from the first 24 hours will be presented.

A hematocrit was performed on an aliquot of the sample taken for fibrinolytic activity. The clot was removed in 24 or 48 hours, leaving in the tube variable amounts of residual cells and serum.

The value for the residual cells obtained by this method in several hundred determinations on normal individuals and patients without coagulation defects was less than 15 per cent of the total cells as calculated from the hematocrit for each determination. When 25 per cent or more of the total cells were found in this fraction, it was felt that fibrinolysis had occurred. The term "per cent lysis" is used to denote the per cent of the total red blood cells remaining in the tube after the clot is removed. Any reading below 20 per cent was not interpreted as being due to lysis. When complete lysis of the clot occurred (100 per cent), all the cells were in the residual fraction. Although the method is obviously semi-quantitative, it provided a practical tool for the estimation of the sum total of fibrinolytic activity induced by the SK in the patient's undiluted, whole blood, together with all its inhibitors and accelerators, and without the addition of any extraneous materials except those contained in Varidase®.

Serum inhibitor. Although by its usage "inhibitor" implies inhibition by a specific substance, in the tests performed the particular constituent in whole blood which is responsible for the inactivation of the streptococcal fibrinolytic system has not yet been identified. Furthermore, it was not clear which part or parts of the streptococcal fibrinolytic process were inhibited. The results of the inhibitor tests represent, therefore, a broad inhibitory phenomenon which may involve one or more factors.

The inhibitory effect described in this article as inhibitor was destroyed by heating at 56°C. for 2 to 6 hours, unlike specific anti-SK antibody which is heat stable at 56°.

The inhibition test was performed as follows: Serial dilutions of a solution of SK were made so that amounts

[3] Normal serum albumin (human) salt-poor, U.S.P., Cutter Laboratories, Berkeley, California.

[4] Chlor-trimeton® Maleate, brand of chlorprophenpyridamine maleate, Schering Corp., Bloomfield, New Jersey.

[5] Pyribenzamine® Hydrochloride, Brand of Tripelennamine Hydrochloride, Ciba Pharmaceutical Products, Inc., Summit, New Jersey.

[6] DC 200, Dow-Corning Corp., Midland, Mich.

[7] The tip was made by winding five turns of Chromel-A wire, gauge 20, on the tip of a 5 by 38 mm. sheet metal screw. (Chromel-A, Haskins Mfg. Co., Detroit, Mich.)

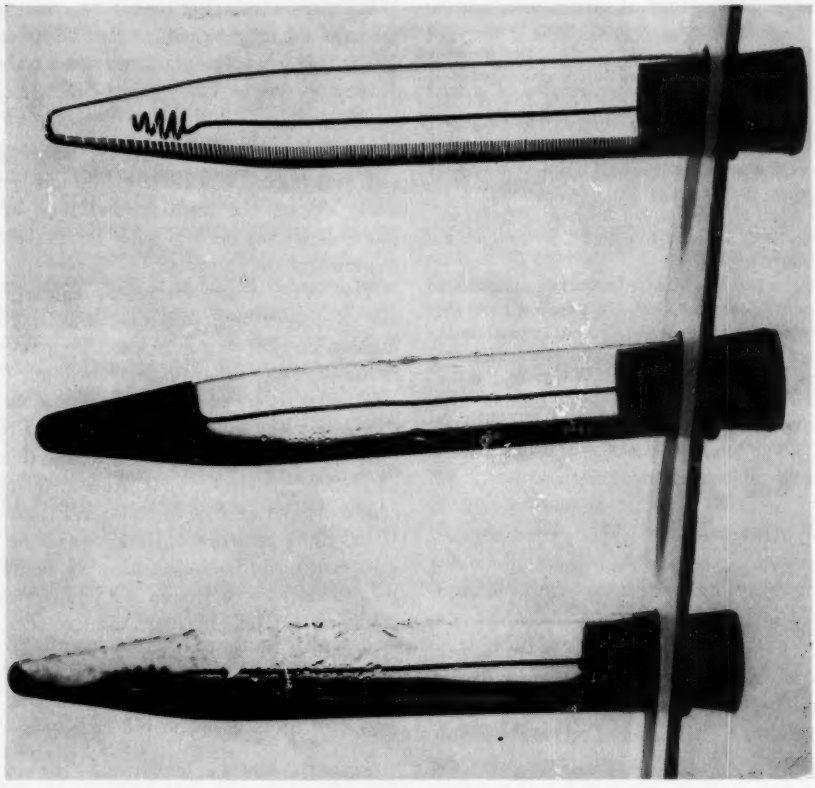

FIG. 1. PHOTOGRAPHIC ILLUSTRATION OF TUBES EMPLOYED IN FIBRINOLYTIC TESTS OF PATIENTS' BLOOD (SEE MATERIALS AND METHODS)

The upper centrifuge tube shows the configuration of the wire to which the clot adheres.

Two ml. of fresh whole blood was added to the centrifuge tube in the middle and two ml. of fresh whole blood (containing SK) was added to the lower tube. The photographs were made after 2 hours incubation at 35°C.

of SK varying from 2,000 units to 10 units were contained in 0.1 ml. of gelatin buffer. One tenth of one ml. of each dilution was then dispensed into 12 by 75 mm. test tubes. Whole blood, obtained by venepuncture with a siliconed syringe was placed immediately in a siliconed flask and 1.0 ml. of blood was added to each tube containing the amount of SK indicated in Figures 2 to 4. After mixing by inversion, the time required for coagulation of the blood was recorded, as was the subsequent time required for complete lysis of the formed clot.

Because of the fact that the mechanism of inhibition is not yet clearly defined, the approximate amount of inhibitory effect found in each patient's blood (Fibrinolytic Profile) has been recorded in Figures 2 to 4 as + (low), ++, +++ (moderate), and ++++ (highest) rather than attempting to employ arbitrary quantitative units.

For purposes of comparison, inhibitor assays were also performed as previously described for plasmin or serum inhibitor (3), and trypsin inhibitor (4), respectively.

Plasminogen. The plasminogen was determined as previously described (3).

Fibrinogen. The method previously described for the determination of fibrinogen (3) was modified for use with the method of Ratnoff and Menzie (6). In the first method, the fibrin was brought down and washed by centrifugation in the cold, in the presence of trypsin inhibitor. In the modified method the plasma was diluted with saline-phosphate buffer at pH 6.4 in an excess of calcium as described by Ware, Guest, and Seegers (7). Further, it was noted that the fibrin adhered more readily to the glass beads if 1 ml. of glass beads .0045 inch in diameter [8] were added gradually during the entire clotting

[8] Minnesota Mining Corp., Minneapolis, Minn.

process. The resulting clot was allowed to remain in the cold for 15 to 20 minutes and more glass beads were added if further clot formation occurred. After washing three times with the cold buffered saline and expressing the clot, the latter was digested in 2 ml. of 1 N NaOH, heated with agitation to 100°C. for 15 minutes, and neutralized with 2 ml. of cold 1 N HCl. The "clottable tyrosine" was then determined (3). Unfortunately, it was not possible to centrifuge the initial sample of citrated whole blood in the cold, but the centrifugation was generally performed in about 10 minutes, after which the plasma was stored at − 20°C.

Complement. The serum complement was determined by the method employed by Fischel, Pauli, and Lesh (8).

Other laboratory determinations. Conventional methods were used for the red blood cell count and hemoglobin, hematocrit, white blood cell count and differential, platelet count (Fonio's method), bleeding time (Duke methods), coagulation time (Lee-White method) (9), and prothrombin time (Quick method with Simplastin®[9]) (10).

RESULTS

Hematological Findings

Erythrocytes, hemoglobin, and hematocrit

The determinations in 11 patients were performed before, during, and after the termination of the infusions. In no instance was any change observed in either RBC counts or amount of hemoglobin. Similarly, the hematocrit reading present before the infusions were begun did not alter during the period of instillation nor for 24 hours after its cessation.

Hemolysis was not observed, nor were immature red cells noted in the peripheral blood smear that might suggest a response to hemolysis.

White blood cell and differential counts

The counts were performed before the infusion was begun, at least two additional ones were made during the infusion and then at varying intervals for the next 24 to 36 hours following the cessation of the infusion.

The WBC count increased regularly and the rise averaged approximately 8,000 WBC, the highest number being reached approximately 20 hours after cessation of the infusions (Table I).

This increase was referable primarily to polymorphonuclear leucocytes and metamyelocyte II's; differential counts performed at this time showed that these two cell types accounted for 80 per cent to 95 per cent of the total WBC count. The WBC count returned to the range of the pre-infusion range within the ensuing 24 hours. These results are in accord with the findings of patients receiving SK–SD in extravascular areas for the eradication of hemorrhagic clots and purulent exudation (11).

Platelets

Since the limits of variation of the method employed in making platelet counts is considerable, the differences were of questionable significance. In four of the eight patients a decrease below the pre-injection count occurred during the period of infusion. In an analysis of 76 determinations

[9] Simplastin®, Chilcott Laboratories, Morris Plains, New Jersey.

TABLE I

Variations in W.B.C. counts, platelet counts, and prothrombin times in association with intravenous infusions of SK

Patient	WBC counts				Platelet counts (*thousands*)			Prothrombin time (*seconds*)	
	Pre-inf. WBC	Highest count WBC	Highest count Time* (*hours*)	Final WBC†	Pre-inf.	During inf. (Lowest)	Post-inf.	Pre-inf.	Post-inf. (Highest)
Wash.	7,900	14,200	22	14,200	268	201	241	15.	22.
Petr.	8,100	11,300	5	6,400	304	314	368	15.2	24.7
Bro.	7,800	12,700	6	5,800	291	138	201	14.3	19.
Bar.	9,800	25,200	20	11,500	523	229	297	15.8	23.8
McD.	14,700	24,800	20	11,500	520	506	360	12.9	18.
Gill.	5,700	30,350	20	8,600	201	200	201	17.2	24.
Bai.	6,500	16,100	25	9,700	464	452	401	14.2	14.2
Woll.	10,700	12,400	1	8,300	401	390	555	14.9	33.4
Cynb.	8,700	12,400	18	8,300	—	—	—	13.6	20.4
Gleas.	—	13,400	12	8,300	—	332	371	18.	20.5

* Refers to post-infusion time (hours) when highest count was obtained.
† Done 24–48 hours, post-infusion.

the changes ranged from an increase of 150,000 to a decrease of 294,000 and averaged 50,000. In no instance did the platelet count fall below 115,000. The fall in platelets was especially prominent in two patients, *Bar* and *Bro*, the decrease averaging 180,000 and 130,000, respectively. It was of interest that these two patients received a dose of 100,000 units of SK, and responded with a relatively mild proteolytic system. However, the platelet count from patients who had larger doses of SK (*McD* and *Bai*) changed relatively little, while the platelet count on *Woll* and *Petr* who had more proteolytic activity for a longer period of time actually increased. In the subsequent counts made on the following day, the number of platelets trended toward the pre-infusion level (Table I). Since no significant changes in bleeding time or clotting time occurred, no abnormalities of blood coagulation that might have been referable to alterations in platelets were observed.

The *coagulation* time generally varied less than 2 to 3 minutes. The changes ranged from an increase of 3.5 minutes to a decrease of 7.5 minutes and averaged 1.5 minutes. The lowest coagulation time recorded was 6 minutes.

The *bleeding* time did not vary more than 1 to 2 minutes in any of the patients.

Although not related to the specific factors that influence tests for bleeding time or clotting time, some oozing of blood occurred in three patients during periods of active fibrinolysis, from the site of frequent stab wounds made in the finger pads for the various hematological tests. This bleeding from punctured cutaneous capillaries occurred only during periods when the circulating blood exhibited 100 per cent degree of fibrinolysis. Even though small clots formed in a normal manner at the sites of the punctures, they subsequently underwent lysis through the action of the fibrinolytic system contained within the coagulum as it formed. By the topical application of pressure, the oozing was controlled.

In a subsequent discussion consideration will be given to the possibilities of bleeding that might occur during periods of active fibrinolysis, *in vivo*.

Effect of infusions on prothrombin time

From the specific figures given in Table I, it may be seen that the prothrombin time was increased moderately following the intravenous infusions of SK and reached a maximum about 8 to 20 hours after cessation of the infusion. A return to normal levels occurred within the ensuing 12 to 24 hours.

Inasmuch as the prothrombin determinations were performed by the one-stage method which does not differentiate between prothrombin and the prothrombin accelerators, further studies are now in progress. Preliminary results of these determinations show a marked fall in Ac-globulin and a mild but definite reduction in the prothrombin.[10] Sherry, Titchener, Gottesman, Wasserman, and Troll reported that Ac-globulin was depressed in dogs following intravenous infusions of SK (12).

In view of the fact that several increments of the blood have exhibited alterations as a result of the proteolytic activity, *in vivo*, in patients, Figures 5 and 6—to be subsequently described—will indicate the concomitant changes in prothrombin time together with plasminogen, fibrinogen, and complement in relation to the presence or absence of fibrinolytic activity.

Urinary findings

The abnormalities noted in repeated examination of urine consisted of: albuminuria irregularly present and slight, the appearance of a few cylindroids and granular casts, a moderate number of WBC, and occasionally 3 to 4 RBC per high power field. The changes are similar to, but less marked than, those observed in patients receiving local injections of SK–SD, who had a pyrogenic response to the treatment (11). The abnormalities appeared a few hours after the infusion was begun. On the day following the termination of the infusion, the proteinuria and cylindruria, when present, had disappeared. Two days post-infusion the urinary findings returned to normal.

Toxic effects

In the present studies it was found that a febrile response of small but definite proportions was associated with the intravenous injection of

[10] These determinations were performed in conjunction with Dr. W. H. Seegers and associates and will be subsequently reported as a separate study.

TABLE II

Effect of intravenous infusions of SK on temperature and blood pressure. Patients were treated with Amidopyrine and Chlor-Trimeton®

Patient	Pre-inf. temp.	Dosage of SK (*units*)	Post-inf. temp. Highest (*F.*)	Post-inf. temp. Occurrence (*hours*)	Pre-inf. B.P. *mm. Hg*	Post-inf. B.P. Lowest *mm. Hg*	Post-inf. B.P. Occurrence (*hours*)	Time return to normal (*hours*)
Wash.	99.2	100,000	100.	10	120/78	98/74	10	24
Petr.	98.6	80,000	100.2	3	120/70	102/60	8	12
Bro.	98.6	100,000	102.	4	126/94	74/54	24	48
Bar.	97.6	100,000	100.2	6	110/70	84/48	24	48
McD.	98.4	125,000	99.6	8	102/70	66/50	12	48
Gill.	98.	100,000	99.2	2	112/60	76/46	14	24
Bai.	98.6	120,000	99.6	6	132/70	92/60	20	48
Woll.*	98.8	100,000	99.8	4*	160/90	130/90	34*	56
Cynb.*	98.8	100,000	100.2	1*	110/64	96/66	14*	30
Gleas.*	98.6	150,000	100.	4*	108/60	90/60	12*	48

* Patients Woll., Cymb., and Gleas. received 2, 3, and 2 repetitions of infusions. Time of recorded highest temp. and lowest B.P. were following 1st inf. No exacerbations of either Temp. or B.P. occurred with additional infusions.

Varidase® containing as little as 5,000 units of SK. As the dosage of SK was raised, the pyrogenic effect became more marked. During the local use of SK–SD (Varidase®) in the treatment of hemothorax and empyema (*2*), it was found that 0.1 gm. of amidopyrine given three times daily on the day prior to and the day of the local treatment modified substantially the febrile response that sometimes occurred as a result of the treatment. Inasmuch as a rise in temperature of 1 to 2°F. occurred 6 to 8 hours after the intravenous infusion in those patients who received 5,000 units of SK (without amidopyrine), and because this febrile response took place even though the SK in the enzyme preparation was specifically neutralized by an excess of anti-SK antibody, the pyrogenic response was thought to be due to a non-specific type of foreign protein reaction. No leukopenic effects associated with the administration of amidopyrine have been observed as may be noted from the data on leucocyte counts contained in Table I.

The febrile response that occurred in 10 patients who received 80,000 to 150,000 units of SK while being treated with amidopyrine was mild (Table II). In patients untreated with amidopyrine, the rise in fever was observed to occur about 6 hours after local injections of SK-SD into the diseased thoracic area, the temperature reached its peak in about 24 hours and subsided during the following 24 to 48 hours.

In the present group of patients the rise of 1 to 2 degrees in temperature reached its peak at an earlier point and had returned to normal the following day. In no instance has the pyrogenic response presented a serious difficulty.

With doses of 80,000 to 150,000 units of SK utilized in this study the hypotensive effect of the infusions has been consistent in its occurrence and the drop in blood pressure has been significant. The blood pressure began to decrease within 3 to 6 hours after the termination of the infusion and the decline continued for the varying periods of hours indicated in Table III until the lowest point was reached. Within the next 4 to 8 hours the pressure began to rise and gradually and progressively increased to its pre-infusion level during the ensuing 24 to 48 hours. During the hypotensive period there were no symptoms of dizziness or impending shock.

The hypotensive effect was more evident in the changes in systolic pressure than in the diastolic pressure, the average drop in systolic readings was 31 mm. Hg points and the diastolic was 14 mm. Hg.

The changes in blood pressure were not correlated with the presence or absence of fibrinolytic activity. For example, in patient *Petr* who exhibited 100 per cent lysis for 8 hours post-infusion and had 25 per cent lysis 48 hours post-infusion, the blood pressure fell about half as much as that in patient *Bai* whose blood had no fibrinolytic activity during the total period of observation (Table II).

Furthermore, two patients (not included among the 11 of this report) with a high anti-SK anti-

body were given 20 to 30,000 units of SK intravenously, without amidopyrine or Chlor-trimeton®; no fibrinolytic activity became evident but a significant fall in blood pressure occurred in each instance.

The chief *subjective symptoms* complained of by the patients were weakness, anorexia and occasionally nausea, which as a rule appeared during the night following the infusion. In a few instances vomiting occurred. The gastric symptoms when present, disappeared the following day.

The Occurrence of Streptococcal Fibrinolysis, in Vivo, in Patients Receiving Intravenous Infusions of SK

In the development of the study the earliest observations indicated that rapid intravenous injections performed in a few minutes time did not result in, *in vivo*, fibrinolysis even though the dosage of SK was above 50,000 units.

Consequently, continuous infusions were instituted according to the procedure that had proved effective in causing the liquefaction of intravascular clots in rabbits (3). As the dosage of SK was gradually increased, the duration of the continuous infusions was varied over a wide range from 2 to 12 hours in an effort to determine the rate of flow that would yield the most effective lytic system.

When the maximum dosage of SK so far administered was reached, with the toxic manifestations appropriately controlled, a plan of procedure was, with certain variations, followed in 11 consecutive patients. The complete data concerning them will be given in Figures 2 to 6 and Tables IV and V.

However, some of the preliminary findings may be briefly noted. As the dosage of SK was increased to 25,000 units and above, sporadic instances of fibrinolytic activity were evident in the circulating blood.

In Table III the dosage of SK and the duration of the infusion are given for a group of individuals whose blood taken, at the termination of the infusion for a single fibrinolytic test exhibited 100 per cent fibrinolysis. Other patients receiving similar dosages did not develop an active system. No other determinations of factors in the fibrinolytic system were made. Consequently the amount and significance of inhibitor, which will be subsequently shown to be of special importance, was not estimated.

TABLE III

Sporadic instances in which 100 per cent fibrinolysis was present in relation to dosage of SK and duration of infusion *

Patient	Dosage of SK	Duration of infusion (*hours*)
Hank.	25,000	6
Who.	30,000	8
Ban.	40,000	13
Gre.	65,000	10
Fer.	80,000	9
Ben.	80,000	6
John.	90,000	6
Nel.	95,000	6
Son.	100,000	6

* Single sample of blood taken at end of infusion.

The data indicate that an active fibrinolytic system may appear in isolated instances with the administration of varying amounts of SK considerably less than the maximum doses employed in this study provided the infusion is continued for several hours. In the patients cited in Table III no determinations were made of the time of appearance of active fibrinolysis during the course of the infusion nor the duration of activity following termination of the infusion.

The Results of Detailed Studies of 11 Patients Receiving 100,000 Units of SK, ± 25,000 to 50,000 units

The data to be described were derived from patients who either received continuous infusions during 4 to 5 hours or were given the total dose by intermittent infusions, with intervening intervals of 1 to 3 and ½ hours between the infusions.

Among all the determinations that were made, the simplest and perhaps the one of greatest practical significance consisted of removing 2 ml. of blood, introducing the sample into the special type of centrifuge tube described in *Materials and Methods*, and incubating it in a water bath at 37.5°C. The time of complete coagulation was noted and was followed by observing the occurrence of fibrinolysis during the subsequent 24 hours or longer. The results of the fibrinolytic tests varied from complete liquefaction, to partial lysis, to no lysis. Furthermore, it was observed that differences in the rate of lysis occurred even

though the ultimate lysis was the same in different tubes. For example, the 100 per cent lysis in one test occurred in 30 minutes whereas in another test with another patient's blood, 100 per cent lysis required 8 to 10 hours incubation. The differences in rate of lysis is not included in the data presented, the results given in the Tables representing the final reading.

Another factor involved in both the rate and completeness of fibrinolysis is related to the quantity of fibrinogen available for transformation into fibrin. During the periods when the lytic system was active within the circulating blood, there was, Figures 5 and 6, a reduction in the amount of circulating fibrinogen which was probably due to intravascular streptococcal fibrinogenolysis. Consequently, in tests, *in vitro*, the amount of fibrin formed from the decreased amount of circulating fibrinogen, is also less than normal, yielding a smaller amount of substrate to be lysed by the SK-plasmin system of the circulating blood.

It soon became evident that the degree of fibrinolysis created in a patient's circulation bore a relation to the amount of inhibitor that was present prior to the beginning of the infusion. The pre-infusion test for "natural" inhibitor has been routinely performed in each patient and will be referred to as the Fibrinolytic Profile of an individual patient since it reflected to a considerable degree the fibrinolytic effects of the subsequent infusion of SK at the dosage employed.

Results of inhibition tests

Figure 2 contains a group of five patients that have been listed as exhibiting a low (+) amount of inhibitor.

From Figure 2 it may be seen that in the tubes containing 100, 60, and 40 units of SK, the most rapid lysis occurred, ranging among the patients in this group from 8 to 12 minutes. With additional dilutions of SK employed (20 and 10 units) the lysis time slowed, but in all of them complete 100 per cent lysis occurred.

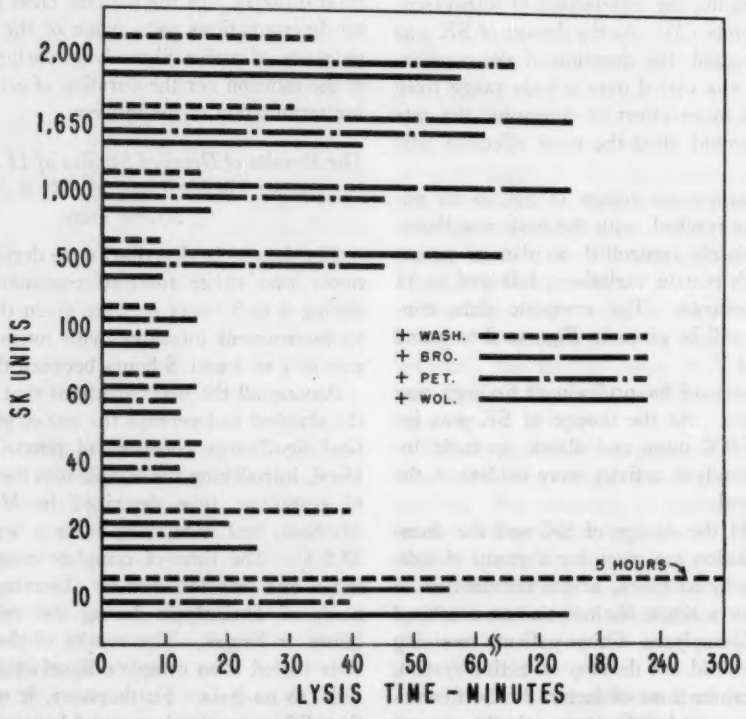

Fig 2: Fibrinolytic Profile of Patients with Low (+) Inhibitor.

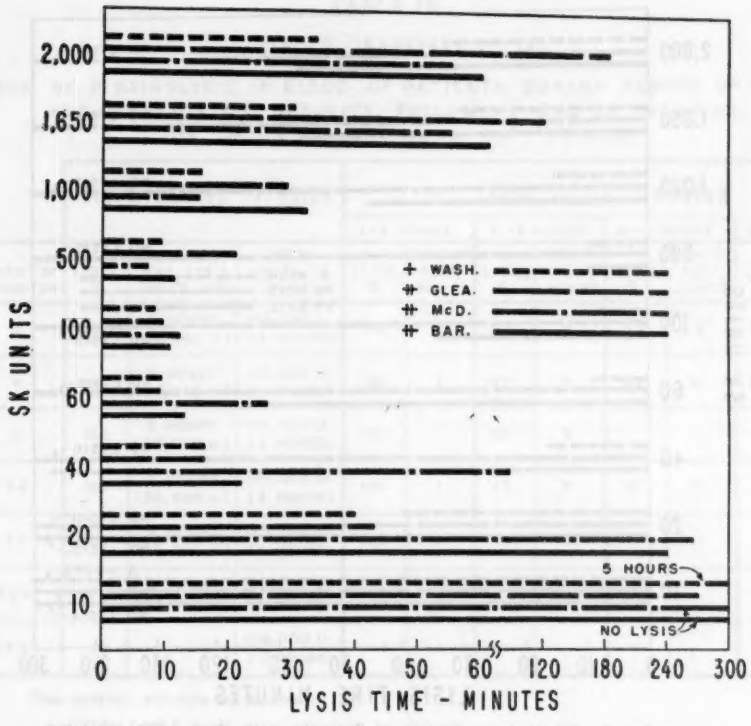

Fig. 3 : Fibrinolytic Profile of Patients with Moderate (++) Inhibitor.

As the amount of SK was increased above 500 units, a slowing of the lysis also occurred. The retarded rate of lysis observed in tests with larger amounts of SK has been interpreted as being due to an inhibitor of SK previously described (2) as being present in high concentrations of SK, the inhibitor being present in the Varidase® preparation itself.

In Figure 3 are the Fibrinolytic Profiles of each of three patients who have been arbitrarily classified as having a somewhat larger amount of inhibitor than the patients included in Figure 1. The increased inhibition is particularly noted in the tubes containing 20 and 10 units of SK, but a slightly longer lysis time is also noted in all the tubes containing 40 units. The results with Patient *Wash*, one of the low inhibitor group, is reproduced again in Figure 2 with a dotted line in order to indicate the comparative difference between the two groups.

Figure 4 contains the Fibrinolytic Profile of two patients rated as having high (++++) inhibitor. The blood of Patient *Bai* exhibited no lysis with any of the dilutions of SK and the blood of Patient *Cous* yielded only partial lysis in dilutions of SK that have proved in tests with other patients' blood to give maximum rates of liquefaction. The findings of Patient *Wash*, of the low inhibitor group, are also included in Figure 4 for purposes of comparison.

Occurrence and Duration of Active Fibrinolysis in the Blood of Patients

The findings recorded in Tables IV and V deal with the time of appearance of maximal fibrinolytic activity as it occurred during the course of the infusion and also the duration of activity after cessation of the infusion. Table IV concerns patients who had a single infusion; Table V concerns patients who had either two or three successive periods of infusion.

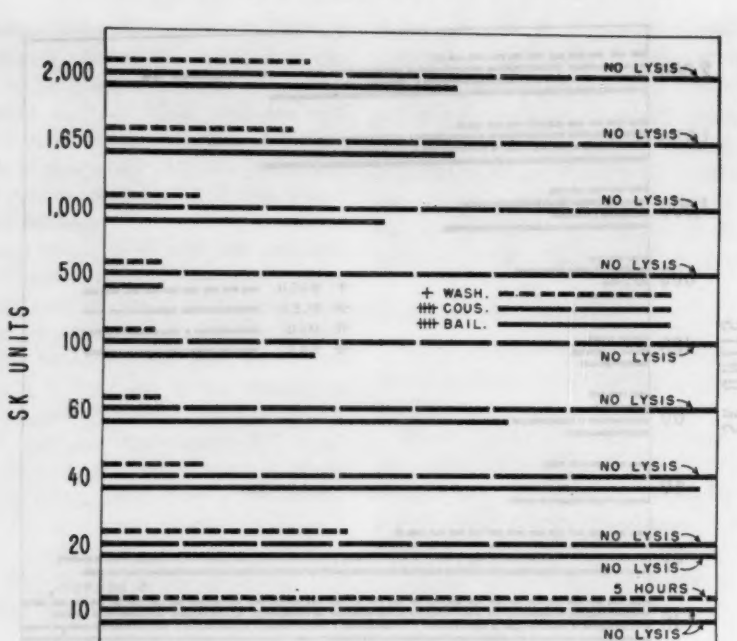

Fig. 4: Fibrinolytic Profile of Patients with High (+++) Inhibitor.

For Patient *Wash* (listed at the top of Table IV) it may be noted that the amount of inhibitor is classified in the low (+) group. As the tabulated data indicate, this patient's blood exhibited complete lysis after 50,000 units of SK had been introduced during the first 2 hours. When the infusion of 100,000 units had been completed in 4 hours, tests during the post-infusion period demonstrated that 100 per cent lysis persisted for 1 hour and was subsequently followed by a progressive decrease in the completeness of lysis of the test clots until, at the end of 24 hours, the patient's blood had returned to the near normal range.

From the data of the other six patients in Table IV, the relationship between the amount of inhibitor and the amount of SK required to initiate an active system are indicated as well as the effect of the larger amounts of inhibitor in shortening the post-infusion period of lytic activity.

In view of this finding, therefore, an additional group of four patients received repeated infusions with intervals of 1 to 3 hours between treatments. The results are given in Table V.

The first two patients of Table V, *Woll* and *Cynb*, had low amounts of inhibitor. In them the total dose of 100,000 units of SK was divided between 80,000 units and 75,000 units, respectively, at the first treatment, and 20,000 units and 25,000 units in the second period of infusion. The results suggest that the second dose, which was smaller than the first, represented a "booster" dose since the duration of post-infusion lysis (42 to 44 hours) was longer than in any of the patients of Table IV who received 100,000 units in a single infusion with the exception of Patient *Petr* of Table IV.

Further evidence for the effectiveness of a "booster" dose was obtained in three cases not represented here because of some incompleteness in the data. In two of these, the fibrinolysis induced by the initial dose of 50,000 to 75,000 units of SK was no longer evident 2 to 4 hours after the initial infusion. When, however, a smaller

TABLE IV

SINGLE INFUSIONS OF SK.

OCCURRENCE OF FIBRINOLYSIS IN BLOOD OF PATIENTS DURING PERIOD OF INFUSION, AND DURATION OF ACTIVITY, FOLLOWING END OF INFUSION.
Its Correlation with Amount of Inhibitor.

		LYSIS DURING INFUSION			LYSIS AFTER INFUSION							
					1-3 HOURS		4-8 HOURS		8-12 HOURS		12-36 HOURS	
PATIENT	* AMOUNT OF INHIBITOR	LYSIS, %	TIME OF MAX. LYSIS (UNITS SK)	TOTAL UNITS SK (TIME)	LYSIS, %	TIME OF TEST, HOURS	LYSIS, %	TIME OF TEST, HOURS	LYSIS, %	TIME OF TEST, HOURS	LYSIS, %	TIME OF TEST, HOURS
WASH.	+	100	2 HOURS (50,000 U.)	100,000 U. (4 HOURS)	100	1	50	4	40	9	20	24
PETR.	+	100	2 HOURS (40,000 U.)	80,000 U. (4 HOURS)	100	1	100	5	85	10	50	30
BRO.	+	100	3 HOURS (60,000 U.)	100,000 U. (5 HOURS)	100	1	30	5			15	17
BAR.	++	100	2 HOURS (50,000 U.)	100,000 U. (4 HOURS)	100	1	30	4	15	8		
McD.	++	100	4 HOURS (125,000 U.)	125,000 U. (4 HOURS)	100	3	10	8				
GILL.	+++	45	4 HOURS (80,000 U.)	100,000 U. (5 HOURS)	<50	2						
BAI.	++++	10		120,000 U. (4 HOURS)	10	1						

*See Inhibitor, Materials and Methods

TABLE V

MULTIPLE INFUSIONS OF SK.

OCCURRENCE OF FIBRINOLYSIS IN BLOOD OF PATIENTS DURING PERIOD OF FIRST INFUSION, AND DURATION OF ACTIVITY FOLLOWING END OF LAST INFUSION.
Its Correlation with Amount of Inhibitor.

			LYSIS DURING DIVIDED INFUSIONS			LYSIS AFTER INFUSION									
						1-3 HOURS		4-8 HOURS		8-20 HOURS		20-48 HOURS		48-72 HOURS	
PATIENT	* AMOUNT OF INHIBITOR	INFUSION	LYSIS, %	TIME OF MAX. LYSIS (UNITS SK)	TOTAL UNITS SK (TIME)	LYSIS %	TIME OF TEST, HOURS	LYSIS, %	TIME OF TEST, HOURS	LYSIS, %	TIME OF TEST, HOURS	LYSIS, %	TIME OF TEST, HOURS	LYSIS, %	TIME OF TEST, HOURS
WOLL.	+	1st	100	2 HOURS (40,000 U.)	80,000 U. (4 HOURS)										
		2nd (2 1/2 hours after 1st)	100		20,000 U. (1/2 HOUR)	100	1 1/2	100	5	80	12	60	42	15	72
GYNB.	+	1st	100	2 HOURS (50,000 U.)	75,000 U. (3 HOURS)	100	1	70	2						
		2nd (3 1/2 hours after 1st)	100		25,000 U. (1 HOUR)	100	1	45	4	25	20	25	44	10	68
GLEAS.	++	1st	25	5 HOURS (100,000 U.)	100,000 U. (5 HOURS)	25	1								
		2nd (1 1/4 hours after 1st)			25,000 U. (3/4 HOURS)	100	1								
		3rd (3 1/2 hours after 2nd)			25,000 U. (1 HOUR)	100	1/4			20	14	35	20	30	48
COUS.	++++	1st	0		100,000 U. (12 HOURS)										
		2nd (2 hours after 1st)	0		50,000 U. (5 HOURS)	0		0		0		0		0	

*See Inhibitor, Materials and Methods

dose of 12,000 to 25,000 units of SK was subsequently given, 100 per cent lysis occurred for the ensuing 6 to 10 hours. In the third case, a strongly proteolytic system (ranging from 50 to 100 per cent lysis) was maintained *in vivo* for 72 hours by a total of 57,000 units of SK given in an initial dose of 25,000 units, followed over the three-day period by three divided doses of approximately 10,000 units each.

The last patient of Table V, Patient *Cous* had such a high amount of inhibitor that even a total of 150,000 units given in two infusions failed to create any fibrinolytic activity.

Effect of Infusion of SK on Fibrinogen, Plasminogen and Complement in Circulating Blood

In the previous report (1) it was demonstrated that substantial decreases occurred in the fibrinogen content of inflammatory pleuritic effusions following the injection into the pleural cavity of SK–SD. Furthermore, that SK acts upon fibrinogen as well as fibrin, *in vitro,* has been demonstrated (13). In the studies of intravenous injections of SK into rabbits (3) moderate decreases in the circulating fibrinogen were noted. The decrease proceeded at a slow rate in spite of the constant infusion and the presence of an active fibrinolytic system. The results suggested that the rate of fibrinogenolysis, *in vivo,* may be slower than *in vitro,* or that the reformation of fibrinogen by rabbits may be sufficiently rapid to parallel closely the slow rate of lysis. The latter possibility was further suggested by the finding that on the day following the infusion in rabbits the fibrinogen rose to higher levels than that noted prior to the infusion, indicating the active synthesis of fibrinogen.

Concerning plasminogen (referred to in the previous article (1) as activatable fibrin-lysing system), it was shown in inflammatory pleural effusions that this plasma factor decreased rapidly after the intrapleural injection of SK–SD although the blood level remained unchanged. In rabbits following the intravenous instillation of 1,800,000 units of SK for 24 hours, it was found that the level of plasminogen progressively decreased from 300 units to 30 units per ml. at the end of the infusion.

Pillemer, Ratnoff, Blum, and Lepow have reported (14) that SK added to human serum, followed by the development of plasmin activity, destroyed complement, *in vitro.* The SK-plasmin preparations caused inactivation, particularly of complement components C2 and C4, and also varying amounts of C1.

Samples of serum derived from the serial bleedings of patients before, during and after the infusions of SK, have been tested for their content in complement.[11]

In the subsequent Figures 5 and 6 concomitant alterations in prothrombin time, complement, plasminogen, and "clottable tyrosine" (fibrinogen) are correlated with the fibrinolytic activity created in the general circulation of the patients by the intravenous infusions of SK. Figure 5 contains the findings in a patient with low (+) inhibitor; Figure 6 those of a patient with high (++++) inhibitor.

With respect to the increments of the blood directly involved in streptococcal fibrinolysis, there was a beginning decrease in both fibrinogen and plasminogen at about the time that 100 per cent fibrinolysis was attained and the point of greatest decrease occurred after maximum lytic activity had been sustained for several hours (Figure 5, patient with low inhibitor; Figure 6, patient with high inhibitor). In the post-infusion period the values for these two substances gradually returned to normal as the fibrinolytic activity disappeared.

The sharp drop in complement resulting from the infusion is also evident, as is also a moderate prolongation of the prothrombin time during the post-infusion period (Figure 5).

In Patient *Bai* with the highest amount of inhibitor and in whom no active fibrinolytic system was developed, no quantitative changes occurred in "clottable tyrosine" (fibrinogen), and the prothrombin time remained unchanged throughout and after the infusion (Figure 6). However, a definite decrease occurred in both complement and

[11] The quantitative determinations of complement reported in this article were performed in the laboratory of Dr. David P. Earle and under his direction. The tests for complement in our patients were done in connection with other studies being conducted by Dr. Earle on changes in complement in patients with different diseases. The findings in the SK treated patients are included with the permission of Dr. Earle.

plasminogen, in spite of the fact that no activity of the fibrinolytic system was measurable.

Among the other patients the pattern of the changes as demonstrated in Patient *Wash* (Figure 5) with low inhibitor and in Patient *Bai* (Figure 6) with high inhibitor, have been relatively uniform and may be summarized as follows: The extent to which fibrinogenolysis occurred paral-

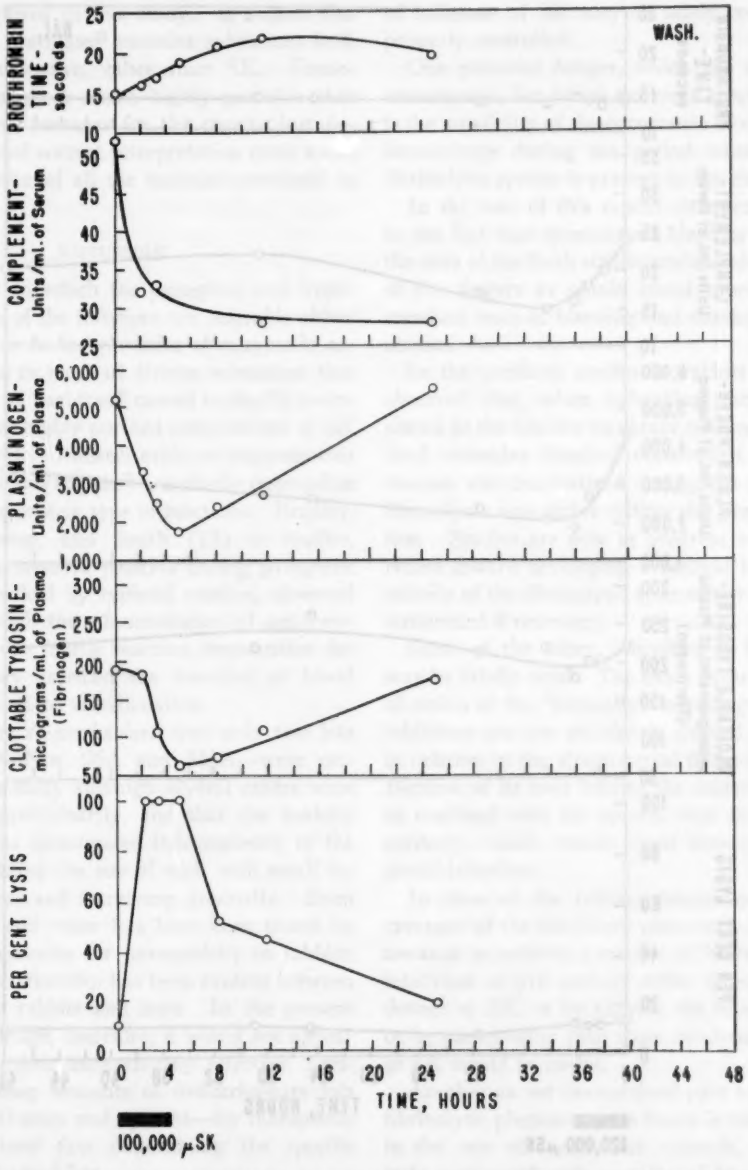

FIG. 5. EFFECT ON CERTAIN BLOOD COMPONENTS OF 100,000 UNITS OF SK INJECTED BY CONTINUOUS INTRAVENOUS INFUSION IN 4 HOURS IN A PATIENT WITH LOW INHIBITOR

leled the degree of activity of the fibrinolytic system. Variations in the prolongation of prothrombin time, which were never great, also conformed to the degree of activity of the lytic system.

However, as noted in Figure 6 substantial decreases in plasminogen and complement occurred irrespective of whether or not active fibrinolysis developed as a result of the infusions of SK.

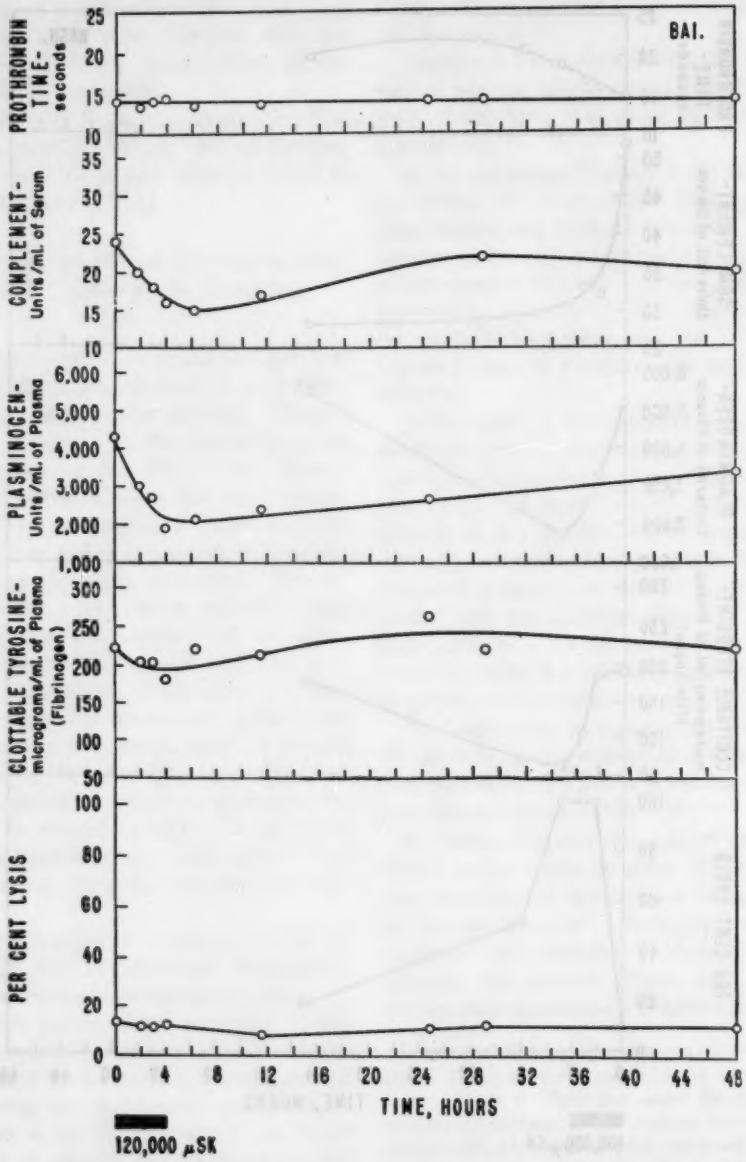

FIG. 6. EFFECT ON CERTAIN BLOOD COMPONENTS OF 120,000 UNITS OF SK INJECTED BY CONTINUOUS INTRAVENOUS INFUSION IN 4 HOURS IN A PATIENT WITH HIGH INHIBITOR

In the light of present knowledge it would be premature to attempt to interpret the findings just described except to point out the somewhat unique spectrum of proteolysis that is associated with the intravenous infusion of SK into patients in the amounts employed in this study. It should also be noted that Varidase® contains substances both known and unknown, other than SK. Consequently, although it seems highly probable that the SK system accounts for the results just described, the final correct interpretation must await the identification of all the moieties contained in Varidase®.

DISCUSSION

The extent to which the pyrogenic and hypotensive effects of the infusions are referable either to SK itself or to the products of enzymatic action, or to one or more of diverse substances that are contained in Varidase® cannot be finally evaluated until more highly purified preparations of SK become available. Indirect evidence suggests that the impurities in Varidase® are chiefly responsible for the foreign protein type of reactions. Bradley, Chasis, Goldring, and Smith (15) in studies, chiefly of hypertensive patients during pyrogenic reactions provoked by typhoid vaccine, observed that even though the administration of amidopyrine obviated the febrile reaction, hypotension developed. They ascribed the lowering of blood pressure, in part, to vasodilatation.

It should be re-emphasized that only two lots of Varidase®—Nos. 96A and 333A—were employed in this study although several others were investigated preliminarily, and that the toxicity of each lot was investigated independently of the other by initiating the use of each with small intravenous doses and increasing gradually. Even though these and other lots have been tested by Lederle Laboratories for pyrogenicity in rabbits, no clear-cut relationship has been evident between the effects on rabbits and man. In the present state of knowledge, therefore, it would not appear advisable to inject intravenously effective, fibrinolysis-producing amounts of indiscriminate lots of SK—25,000 units and upward—for therapeutic purposes without first determining the specific effects of individual lots.

The active fibrinolysis created within the circulating blood exhibited features which indicate that it may serve as an effective method of therapy in the treatment of intravascular occlusions of fibrinous composition. It is also apparent from the data that a considerable amount of additional information is required before the practical therapeutic use of infusions of SK may be safely conducted and properly controlled.

One potential danger, which has not yet been encountered, but which warrents special comment, is the possibility of the occurrence of extravascular hemorrhage during the period when the active fibrinolytic system is present in the circulation.

In the text of this report attention was called to the fact that spontaneous bleeding occurred at the sites of the fresh stab wounds made in the pads of the fingers to obtain blood counts, although standard tests of bleeding and clotting time were normal.

In the previous studies in rabbits (3) it was observed that when indwelling catheters were placed in the bladder to obtain specimens of urine, local excessive bleeding occurred if the bladder mucosa was traumatized during the period when fibrinolysis was active within the general circulation. Studies are now in progress which are directed toward developing a method by which the activity of the fibrinolytic system may be promptly terminated if necessary.

Some of the other difficulties to be overcome may be briefly cited. The exact nature and modes of action of the "naturally" occurring inhibitor or inhibitors are not yet clearly defined, particularly in relation to the streptococcal fibrinolytic system. Because of its heat lability the inhibitor is not to be confused with the specific heat stable anti-SK antibody, which results from hemolytic streptococcal infections.

In view of the relative frequency of the occurrence of the inhibitory phenomenon in varying amounts in patients, a method of "neutralizing" the inhibition of lytic activity either by increasing the dosage of SK, or by altering the rate of infusion, or by means other than those involving the dosage of SK would be useful.

Another as yet unexplained part of the *in vivo* fibrinolytic phenomenon is found in the differences in the rate of lysis. For example, in different tests performed with samples of blood from different patients, 100 per cent lysis occurred in each of the tests but the time required for com-

pletion varied from 30 minutes to 10 hours. Similar variations in rate were observed in tests in which varying degrees of partial lysis occurred. since it is not yet established in patients what degree or rate of fibrinolysis is most advantageous in abolishing pathologically induced intravascular fibrin clots, the significance of the variations in terms of practical effective therapy requires more definition.

Several investigators have injected intravenously a preformed plasmin, activated by SK *in vitro,* into animals, and observed fibrinolysis, *in vivo.*

Cliffton, Cannamela, and Grossi (16) injected a solution containing SK activated human plasminogen into dogs and rabbits and noted a marked increase in the lytic activity of the blood of both animal species. Subsequently the same authors (17) reported on the successful liquefaction by SK-human plasmin of thrombi produced in the femoral veins of dogs. There was a marked increase in the lytic activity of the blood in the general circulation of the animals.

Sherry has elucidated several features of SK fibrinolytic activity which have a bearing on the use, *in vivo,* of SK. This investigator has recently reported that SK in the presence of small amounts of the human serum factor (plasminogen or SK-plasmin) activates bovine or canine plasminogen which exists as a contaminant of the preparations of animal fibrinogens employed in tests performed, *in vitro* (18). According to these findings, therefore, when SK-human plasmin is injected intravenously into dogs, the high degree of fibrinolytic activity developed within the circulating blood of the dog is referable to a considerable degree to the activation of the dog's own system rather than to the direct action of the SK-human plasmin.

In other articles, Sherry, Titchener, Gottesman, Wasserman, and Troll (12, 19) have reported upon the enzymatic dissolution of experimental arterial thrombi in dogs. The authors reported that the injection of moderately small amounts of SK plus partially purified human plasminogen preparations effected rapid activation of the dogs fibrinolytic system with lysis of the arterial clots together with a significant increase in broad proteolytic activity within the circulating blood. Injections of SK alone intravenously in the dogs also promoted clot dissolution but the required amount of SK was greater than that contained in the SK-human plasmin preparation. Minor increases in circulating proteolytic activity in association with marked fibrinolytic activity were demonstrable when SK alone was employed in dogs.

Extensive studies have not been performed to delineate the extent of the range of proteolytic activity occurring in the general circulation of the patients receiving SK. However, the findings suggest that with the dosage of SK employed in the patients, the degree of proteolysis occupied a position intermediate between the "burst" of proteolysis obtained by injections of SK-human plasmin into dogs and the less extensive changes resulting from injections of SK alone into dogs (12, 19).

SUMMARY

Streptokinase (contained in Varidase®), in amounts ranging from 75,000 to 150,000 units, has been introduced intravenously into patients, 11 of whom were studied in detail as described in this article. Both continuous and intermittent infusions of 4 to 5 hour duration have been used.

The hematological effects have consisted chiefly of polymorphonuclear leucocytosis, and some irregular variations in platelet counts with no appreciable alteration of the clotting time or bleeding time. Moderate prolongations of prothrombin time occurred following the creation of active fibrinolytic systems in patients with low titres of naturally occurring inhibitor. No alterations in prothrombin time were observed in patients with high inhibitor in whom active fibrinolysis did not develop.

The principal toxic manifestations were pyrogenic reactions which were well controlled by the administration of amidopyrine, and hypotensive effects for which Chlor-trimeton® Maleate was given. The decreases in blood pressure were usually maximal 8 to 24 hours after termination of the infusion and usually returned to normal during the following 24 hours. The systolic blood pressure was affected more than the diastolic. No symptoms attended the lowering of the blood pressure.

An active fibrinolytic system was developed in six of the seven patients who received continuous infusions for 4 to 5 hours, and in five of the seven, 100 per cent fibrinolytic activity was at-

tained. After termination of the infusion, lytic activity remained 100 per cent for 1 to 3 hours and then decreased with varying degrees of partial activity persisting for 4 to 30 hours depending to a considerable degree on the amount of "natural inhibitor" present in the individual patient's blood.

Repeated infusions (two times in three, and three times in one patient) were given in which the 2nd or 3rd infusion represented a "booster" dose. The degree and duration of fibrinolytic activity was greater and persisted longer in three of the four patients of this group than in those receiving a single infusion even though the total amount of SK was essentially the same.

When an active lytic system was developed in patients with low inhibitor concomitant decreases occurred in fibrinogen, plasminogen, and complement. When no lytic activity occurred in patients with high inhibitor, no fibrinogenolysis or fibrinolysis occurred, but there were significant but lesser decreases in both plasminogen and complement.

The potential hazards present during the periods of active fibrinolysis have been pointed out, and some of the conspicuous problems that remained to be studied have been indicated.

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[12] For comprehensive review of recent articles on clinical use of Varidase® (streptokinase-streptodornase), see bibliography contained in: "Recent advances in Varidase® therapy" distributed by Lederle Laboratories Division, American Cyanamid Co., 30 Rockefeller Plaza, New York 20, N. Y.

THE IODIDE TRAPPING AND BINDING FUNCTIONS OF THE THYROID

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McGinty and Sharp (1) demonstrated that the thyroid glands of rats treated with propylthiouracil retained the ability to concentrate iodide ion but failed to bind the iodine in an organic form. VanderLaan and VanderLaan (2) and Stanley and Astwood (3) showed also that thyroidal iodide, "trapped" under the influence of the antithyroid drugs, could be rapidly discharged from the gland by the administration of thiocyanate ion. Iodine accumulation by the thyroid has therefore been considered a two-step function; the first is concerned with the concentration (trapping) of iodide at a level higher than that in the circulating plasma, while the second, in itself a complicated process composed of several chemical stages, is represented by organic binding of the trapped iodide.

Several authors have inquired into the kinetics of thyroidal iodide trapping and binding (4–6). On the assumption that equilibration of plasma and thyroidal inorganic iodide^{131} (iodide trapping) is instantaneous following intravenous administration of I^{131}, Ingbar (4, 5) calculated that thyroidal binding of trapped iodide proceeded at average rates of 1½ per cent per minute in euthyroid subjects and 6½ per cent per minute in patients with Graves' disease. Wollman (6) assumed that the plasma iodide^{131} concentration remained constant or decreased at a single exponential rate and considered the sequelae of several theoretical alternatives for the relative rates of trapping and binding. In the present study an analysis is presented which permits evaluation of the rates of trapping and binding from observed data without assumptions regarding either process or restrictions in the behaviour of the plasma iodide^{131} concentration. The results of the experimental investigations indicate an extremely rapid binding of trapped iodide in the untreated gland and lead to the conclusion that trapping rather than binding is the rate-limiting step of thyroidal iodine accumulation.

TECHNICAL METHODS

Subjects received intravenous injections of 15 to 200 μc carrier-free NaI^{131} in normal saline solution. Assay of thyroidal radioactivity was performed with a bismuth-walled Geiger counter and a scaling circuit connected to a Streeter-Amet register which automatically printed the cumulative counts at minute intervals, as previously described (7). The sensitivity of this arrangement was 110 counts per minute per μc I^{131} above a background of 60 counts per minute. Five ml. samples of packed erythrocytes, plasma and urine were counted in a well-type scintillation counter with a sensitivity of 1.00×10^6 counts per minute per μc I^{131} above a background of 200 counts per minute. PBI^{131} and iodide^{131} were determined separately utilizing the plasma/RBC ratio of I^{131} as previously described (8, 9).

Extrathyroidal radioactivity "seen" by the counter was determined, for each subject, from the counting rate over the neck following discharge of trapped thyroidal iodide (binding inhibited by Tapazole®) by the intravenous administration of 1.0 gm. NaSCN. Corrections for extrathyroidal activity at all times could then be made according to methods previously described (7). In euthyroid subjects, whether or not binding was blocked, the counting rate during the second minute following intravenous injection of NaI^{131} was usually in excellent agreement with the extrathyroidal radioactivity determined by the method described above. These observations confirm the premise, previously made (7), that there is virtually no thyroidal I^{131} accumulation this early in normal glands. In hyperthyroid subjects, however, appreciable amounts of radioactivity were occasionally present in the thyroid even during the first few minutes so that the extrathyroidal level was sometimes below the earliest recorded counts. The geometry of the counting system was such that the extrathyroidal radioactivity "seen" by the counter was 12 to 13 per cent of the total extrathyroidal radioactivity in almost all subjects, which is in agreement with the total neck activity recorded by this arrangement in athyreotic subjects.

In one group of hyperthyroid subjects, "binding" and "trapping" studies[1] were carried out on the same day in the following manner. Determinations of radioactivity in thyroid and blood were made over a 30 to 35-minute period following the intravenous injection of a small tracer dose of NaI^{131} (15 to 20 μc). Fifty milligrams of Tapa-

[1] "Trapping" study refers to a tracer experiment performed during Tapazole® inhibition of binding. "Binding" study refers to a tracer experiment performed in the absence of Tapazole® inhibition.

zole® (1-methyl 2-mercaptoimidazole) were then given by mouth and 60 to 90 minutes later a repeat study was performed with a dose of I^{131} about 10 to 15 times as great as the first. Observations of thyroidal and blood radioactivities were again obtained and appropriately corrected for the small residual activities of the first dose. One-half to two hours after administration of the second tracer, 1.0 gram NaSCN was injected intravenously.

ANALYSIS

Thyroidal iodide trapping

When organic binding of iodide is completely blocked with adequate doses of Tapazole®, thyroidal radioactivity increases with time reaching a peak value within a few minutes to an hour or two following intravenous injection of NaI^{131}, and then declines parallel with the plasma concentration until all the radioactivity is cleared from the body by renal excretion (Figure 1). Under these conditions the rate of removal from the body is directly dependent on the renal plasma iodide clearance rate and inversely related to the fraction of total iodide trapped by the thyroid. That thyroidal I^{131} is not organically bound but is in equilibrium with plasma iodide is indicated by the parallelism of thyroidal and plasma curves of radioactivity (Figures 1, 2) and by the ready dischargeability of thyroidal radioactivity with NaSCN (Figure 2). If the cumulative urinary excretion of I^{131} is subtracted from the dose administered, the trapped thyroidal iodide^{131} at equilibrium may be expressed as the thyroidal fraction of the dose retained in the body (fr I^{131}_{ret}). The extent of trapping may also be expressed as liters equivalent of plasma content ("thyroidal iodide space"), or as milliliters plasma equivalent per gram of thyroid tissue (thyroid/plasma ratio).

Accumulation of iodide by the thyroid trap could usually not be described adequately by a single exponential rate, since the rate of approach of thyroidal radioactivity to the asymptotic value was generally much more rapid immediately following injection than subsequently (Figure 2). It is not necessary to postulate several thyroidal compartments equilibrating with plasma at different rates to ex-

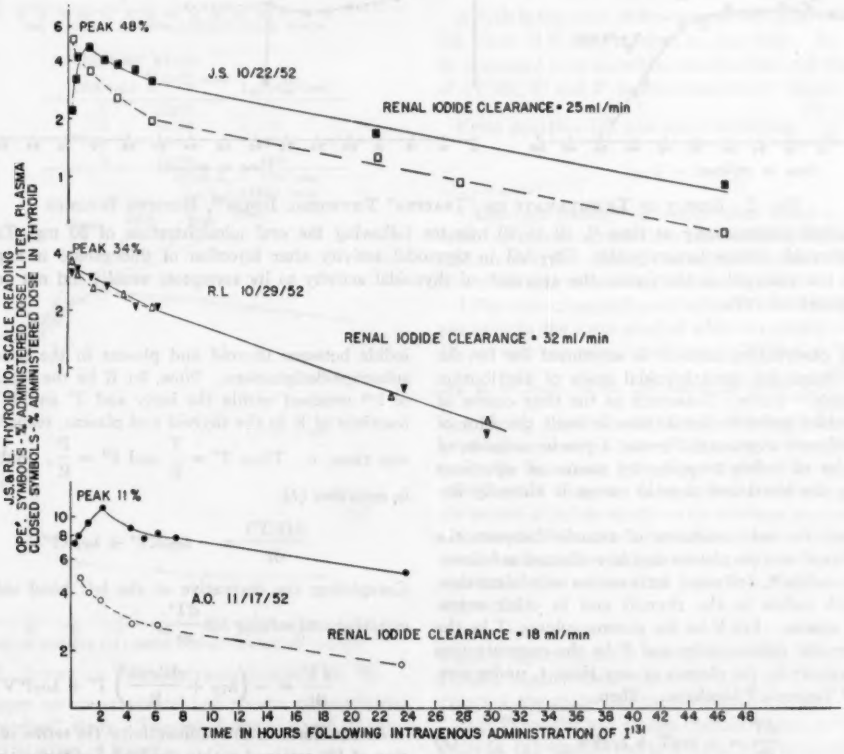

PEAK 48%
J.S. 10/22/52
RENAL IODIDE CLEARANCE • 25 ml/min
PEAK 34%
R.L. 10/29/52
RENAL IODIDE CLEARANCE • 32 ml/min
PEAK 11%
A.O. 11/17/52
RENAL IODIDE CLEARANCE • 18 ml/min
J.S. & R.L. THYROID 10x SCALE READING
OPEN SYMBOLS - % ADMINISTERED DOSE / LITER PLASMA
CLOSED SYMBOLS - % ADMINISTERED DOSE IN THYROID
TIME IN HOURS FOLLOWING INTRAVENOUS ADMINISTRATION OF I131

FIG. 1. CHARACTERISTIC THYROIDAL AND PLASMA RADIOACTIVITY CURVES IN SUBJECTS RECEIVING 25 MG. TAPAZOLE® EVERY 6 HOURS

R. L. and J. S., hyperthyroid; A. O., hyperthyroid but had received I^{131} therapy 2½ months previously. The slopes of the curves can be correlated roughly with the rates of the renal clearance.

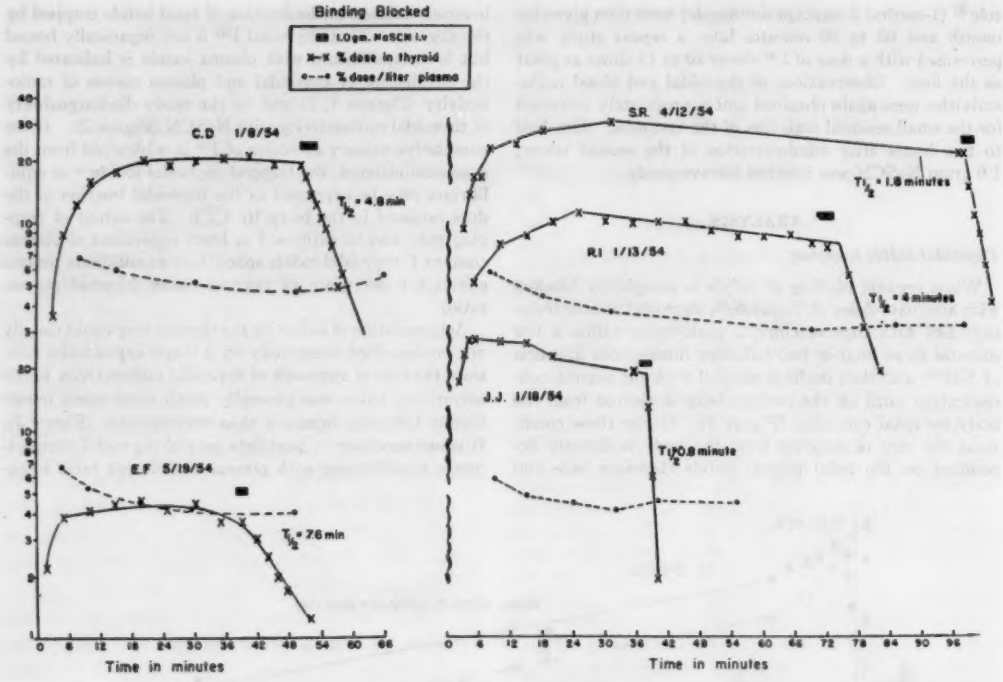


FIG. 2. EFFECT OF THIOCYANATE ON "TRAPPED" THYROIDAL IODIDE^{131}, BINDING BLOCKED

I^{131} injected intravenously at time 0, 60 to 90 minutes following the oral administration of 50 mg. Tapazole®. E. F. euthyroid; others hyperthyroid. The fall in thyroidal activity after injection of thiocyanate is exponential. Although not analyzed in the figure, the approach of thyroidal activity to its asymptote usually did not occur at a single exponential rate.

plain this observation since it is accounted for by the gradually increasing extrathyroidal space of distribution of the iodide^{131} tracer. Inasmuch as the time course of extrathyroidal iodide^{131} distribution is itself the sum of several different exponential terms, a precise solution of the kinetics of iodide trapping by means of equations describing the blood and thyroid curves is virtually impossible.

However, the rate constants of transfer between the thyroid "trap" and the plasma may be evaluated as follows.

Plasma iodide^{131}, following intravenous administration, mixes with iodide in the thyroid and in other extrathyroidal spaces. Let V be the plasma volume, T be the total thyroidal radioactivity and P be the concentration of radioactivity in the plasma at any time, t, under conditions of Tapazole® blockage. Then,

$$\frac{dT}{dt} = -\lambda_{TP}T + \lambda_{PT}PV \qquad (1)$$

where λ_{TP} and λ_{PT} are the rate constants[2] of transfer of iodide between thyroid and plasma in the order of the subscript designations. Now, let R be the total amount of I^{131} retained within the body and T′ and P′V be the fractions of R in the thyroid and plasma, respectively, at any time, t. Then $T' = \frac{T}{R}$ and $P' = \frac{P}{R}$. Substituting in equation (1),

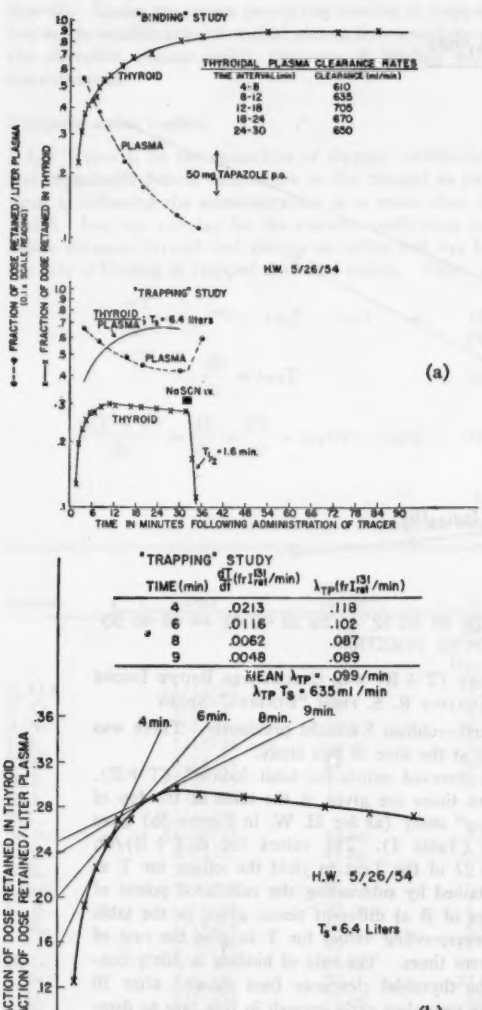
$$\frac{d(RT')}{dt} = -\lambda_{TP}RT' + \lambda_{PT}RP'V \qquad (2)$$

Completing the derivative of the left hand side of the equation and solving for $\frac{dT'}{dt}$,

$$\frac{dT'}{dt} = -\left(\lambda_{TP} + \frac{dR/dt}{R}\right)T' + \lambda_{PT}P'V \qquad (3)$$

At equilibrium, the radioactivity (in terms of the fraction of I^{131} retained within the body) entering the thyroid

[2] During the short term experiments required for equilibration of iodide^{131} with the thyroid trap a steady state with respect to stable iodide^{127} may be assumed. Although this condition is not essential to the analysis, it permits treatment of the transfer rates as constants in the absence of an assumption of first order kinetics for the trapping processes.



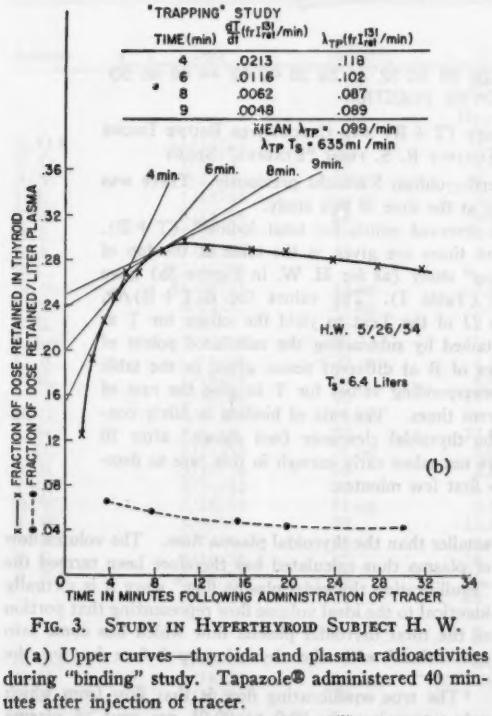


FIG. 3. STUDY IN HYPERTHYROID SUBJECT H. W.

(a) Upper curves—thyroidal and plasma radioactivities during "binding" study. Tapazole® administered 40 minutes after injection of tracer.

Lower curves—"trapping" study. I^{131} tracer injected 60 minutes after the administration of the Tapazole® indicated in the upper half of the figure. The time course for the total thyroid/plasma ratio in liters is also shown. In cases of rapid equilibration this ratio reaches a peak slightly higher than the equilibrium value (T_s). A more precise, slightly lower value for T_s would have been obtained if the study had been continued longer.

(b) Thyroidal and plasma radioactivities of "trapping" study in (a) transferred to a linear plot to permit evaluation of the slopes (dT'/dt) of the thyroid curve. λ_{TP} is then obtained by substitution in equation 6 of the text. In most instances equilibration is more prolonged and dT'/dt may be evaluated at many points up to 30 or 40 minutes or more.

is equal to that leaving. Then, $\frac{dT'_{eq}}{dt} = 0$[3] and

$$\left(\lambda_{TP} + \frac{dR/dt}{R}\right) T'_{eq} = \lambda_{PT} P'_{eq} V \qquad (4)$$

Now let T_s be the thyroidal iodide space in liters equivalent of plasma so that $T_s = \frac{T'_{eq}}{P'_{eq}}$. Then, from (3) and (4), and solving for $\lambda_{TP} + \frac{dR/dt}{R}$

$$\lambda_{TP} + \frac{dR/dt}{R} = \frac{dT'/dt}{P'T_s - T'} \qquad (5)$$

Now, when the rate of loss of I^{131} from the body $\left(\frac{dR/dt}{R}\right)$ is small compared to λ_{TP}, $\frac{dR/dt}{R}$ may be neglected at all times[4] and equation (5) becomes

$$\lambda_{TP} = \frac{dT'/dt}{P'T_s - T'} \qquad (6)$$

dT'/dt is the slope of the curve of thyroidal radioactivity (in terms of fr I^{131}_{ref}) *vs.* time at any time. λ_{TP} can then be evaluated from simultaneous experimental observations of dT'/dt, T' and P' as demonstrated in Figure 3.

From equation (4), and again neglecting $\frac{dR/dt}{R}$,

$$\lambda_{PT} V = \lambda_{TP} T_s \qquad (7)$$

[3] Although iodide[131] excretion does effect a continual lowering of the actual thyroidal radioactivity, the equilibrium situation defined for iodide[131] in terms of *the fraction of the dose retained* is theoretically valid.

[4] The rate of excretion of iodide[131] was 4 to 9 per cent per hour in the cases studied while λ_{TP} ranged from 1½ to 10 per cent per minute as given in Table I. Thus, since λ_{TP} is at least 10 to 100 times as great as $\frac{dR/dt}{R}$, the simplifying approximation inherent in equation (6) may produce an error of 1 to 10 per cent in λ_{TP}. The error generally was about 2 to 3 per cent. This error can be eliminated if frequent urine collections are made during the period of iodide equilibration between plasma and thyroid trap. For cases where this is inconvenient, an alternative analysis which permits evaluation of λ_{TP} with a maximum error of less than 1 per cent is presented in the Appendix.

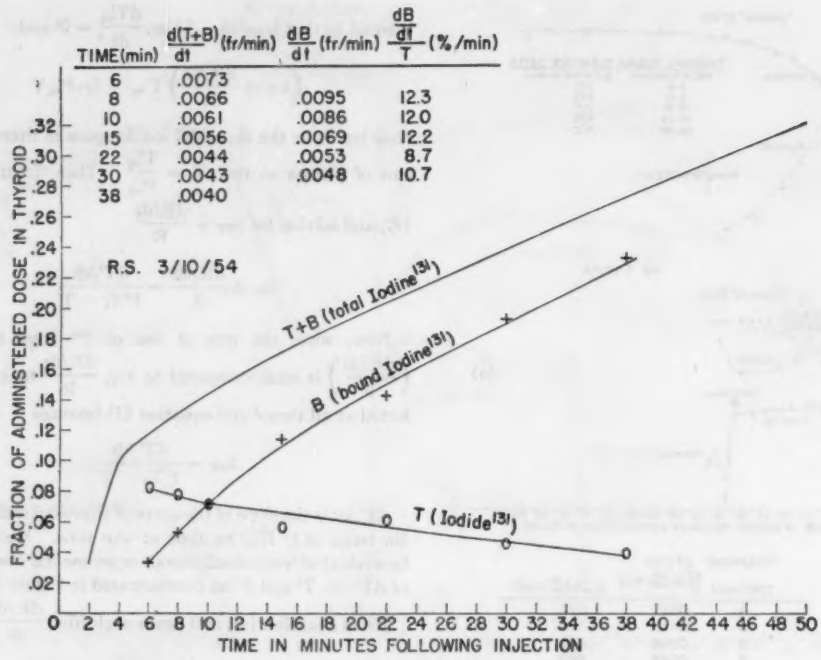


FIG. 4. OBSERVED TOTAL THYROIDAL RADIOACTIVITY (T + B) AND CALCULATED BOUND IODINE (B) AND IODIDE (T) RADIOACTIVITY IN SUBJECT R. S. FROM "BINDING" STUDY

This subject had been treated with I^{131} for hyperthyroidism 5 months previously. There was temporary improvement but patient was in relapse at the time of this study.

A smooth curve has been drawn through the observed points for total iodine^{131} (T + B). The slopes d(T + B)/dt of this curve at different times are given in the table at the top of the figure. Analysis of the curves in the "trapping" study (as for H. W. in Figure 3b) gave $\lambda_{PT}V = 143$ ml. per min. and $\lambda_{TP} = .050$ min.$^{-1}$ (Table I). The values for d(T + B)/dt, $\lambda_{PT}V$ and λ_{TP} were then substituted into equation *11* of the Text to yield the values for T at the corresponding times. Values for B were obtained by subtracting the calculated points of T from corresponding points of (T + B). Slopes of B at different times, given in the table at the top of figure, were then divided by the corresponding values for T to give the rate of binding of trapped iodide (dB/dt/T) at the different times. The rate of binding is fairly constant at about 9 to 12 per cent per minute. The thyroidal clearance (not shown) after 10 minutes was 86 ml. per min. Plasma samples were not taken early enough in this case to demonstrate the higher thyroidal clearance during the first few minutes.

$\lambda_{PT}V$ represents the volume of plasma containing that amount of iodide which is transferred to the thyroid trap per unit time. If equilibrium between plasma and thyroid trap is established during a single circulation through the thyroid, this volume flow represents the thyroidal plasma flow.[5] If the plasma leaving the thyroid has not come into equilibrium with the thyroid trap, then this flow is smaller than the thyroidal plasma flow. The volume flow of plasma thus calculated has therefore been termed the "equilibrating thyroidal plasma flow" since it is virtually identical to the ideal volume flow representing that portion of the total thyroidal plasma flow which has come into equilibrium[6] with the thyroid trap before leaving the

[5] If RBC iodide, which is in rapid equilibrium with plasma iodide, is also available to the thyroid trap during circulation through the thyroid, then the figures for plasma flow may be anything up to 40 per cent too high since at normal hematocrit levels the total iodide content of erythrocytes is about 40 per cent that of the plasma.

[6] The true equilibrating flow is that flow from which only approximately 99.0 to 99.95 per cent of plasma iodide^{131} is transferred to the thyroid trap, assuming thyroid/plasma ratios of 20/1 to 500/1 and thyroidal blood volumes of 20 per cent of thyroid weight. However this is virtually identical to $\lambda_{PT}V$, that flow from which 100 per cent of the iodide^{131} is transferred.

thyroid. Under conditions permitting binding of trapped iodide, the equilibrating thyroidal plasma flow would equal the thyroidal plasma iodide clearance if binding were instantaneous.

Thyroidal iodide binding

Let T and B be the quantities of trapped radioiodide and organically bound radioiodine in the thyroid at any time, t, following the administration of a tracer dose of NaI^{131}. Let λ_{TP} and λ_{PT} be the transfer coefficients for iodide between thyroid and plasma as before and λ_{TB} be the rate of binding of trapped thyroidal iodide. Then

$$\frac{dT}{dt} = \lambda_{PT}PV - \lambda_{TP}T - \lambda_{TB}T \qquad (8)$$

$$\frac{dB}{dt} = \lambda_{TB}T \qquad (9)$$

$$\frac{d(T+B)}{dt} = \frac{dB}{dt} + \frac{dT}{dt} = \lambda_{PT}PV - \lambda_{TP}T \qquad (10)$$

where (T + B) is the total thyroidal radioactivity. It is assumed that negligible amounts of bound iodine have been released from the thyroid or degraded within the thyroid during the period of these studies. Solving for T,

$$T = \frac{\lambda_{PT}VP - \frac{d(T+B)}{dt}}{\lambda_{TP}} \qquad (11)$$

When the values for λ_{TP} (from equation *6*) and $\lambda_{PT}V$ (from equation *7*) obtained from the analysis of the curves during the "trapping" study, are substituted in equation (*11*), T, at any time, is seen to be defined by the plasma concentration of iodide^{131} (P) and the slope of the observed thyroidal radioactivity curve $\left(\frac{d(T+B)}{dt}\right)$ at the same time. Therefore the curve of thyroidal *radioiodide* (T) can be constructed (Figure 4). By subtracting each point on the curve T from the observed total thyroidal radioactivity curve (T + B), the curve for organically bound

TABLE I

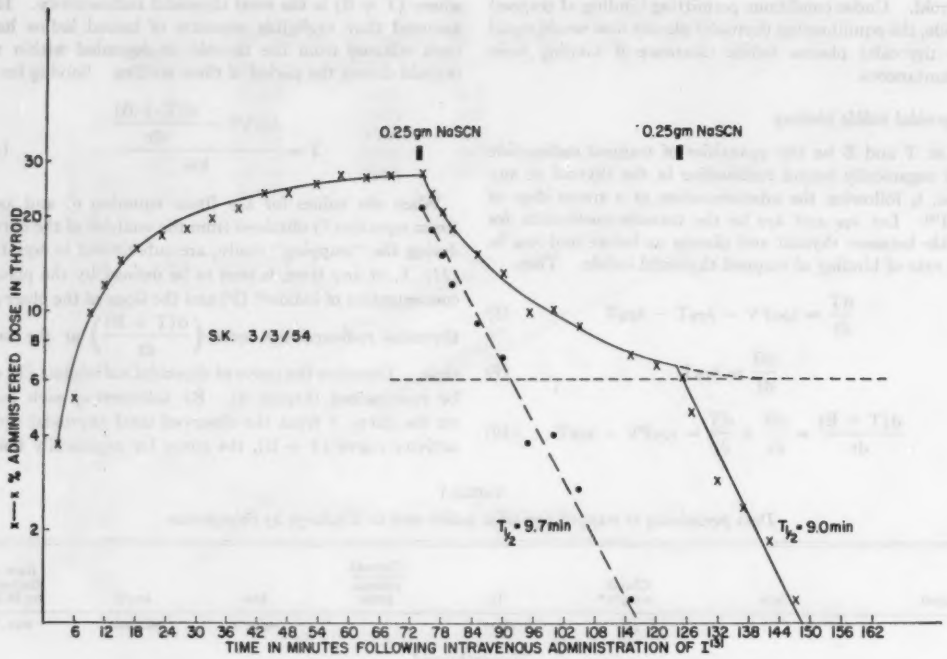
Data pertaining to trapped thyroidal iodide and its discharge by thiocyanate

Subject	Date	Gland weight*	T_s	Thyroid/plasma ratio	λ_{TP}	$\lambda_{PT}V$	Rate of discharge by SCN^-
		gm.	*liters*		*min.*$^{-1}$	*ml./min.*	*min.*$^{-1}$
			Hyperthyroid				
A. O.†	11/17/52	20	3.1	155	d.i.‡	d.i.	—
C. D.	1/8/54	40	4.1	102	.0824	337	.138
A. V.	2/5/54	50 op	29	580	.0290	860	.138
J. J.	1/18/54	44 op	4.9	111	d.i.	d.i.	.865
T. S.	6/14/54	60	4.6	77	.0300	138	.126
R. L.	7/27/53	10	5.4	540	.0277	150	—
R. I.	1/13/54	25	2.75	110	.066	175	.173
S. C.	3/11/54						.200
N. R.	12/30/53	42 op	1.1	26	d.i.	d.i.	2.8
T. L.	6/23/53	41 op	1.57	38	.075	118	—
S. B.	3/5/54	25	2.0	80	d.i.	d.i.	.040
J. Sm.	10/22/52	20	15.7	785	.0155	233	—
C. B.	1/18/54	<20	2.1	>100	.036	76	.144
S. K.	1/18/54	40	8.65	216	.0223	193	.121
	3/31/54	40	10	250	.0215	215	.063
E. G.	1/12/54	94 op	36	382	.026	930	.045
J. St.	1/6/54	80	23.5	294	.015	350	.532
J. U.	7/23/53	31 op	3.9	126	.046	179	—
R. S.	3/10/54	40	2.86	71	.050	143	.107
J. Q.	5/24/54	75	16	214	.0244	390	.030
H. W.	5/26/54	71 op	6.4	90	.099	634	.495
T. W.	3/5/54	30	15.8	526	.035	553	.428
A. K.	7/7/54	20	2.65	132	.033	87	.140
V. C.	3/16/54	25	10	400	.0177	177	.120
R. F-V.	4/26/54	40	34	850	.0206	700	.315
	5/17/54	40	16	400	.0268	430	—
			Euthyroid				
A. O.†	1/20/54	?	2.0	?	d.i.	d.i.	.174
H. F.	1/14/54	15	.83	55	.0470	40	.070
E. F.	5/19/54	20	.94	47	d.i.	d.i.	.087

* Two grams added to weights of glands removed at operation. In those cases not subjected to surgery weights were estimated by palpation. In our hands these estimates are frequently in error by as much as 50 per cent so that the thyroid/plasma ratios are to be regarded as approximate.

† Treated with 4.5 mc. I^{131} 9/3/52; clinically hyperthyroid 11/17/52, clinically euthyroid 1/20/54.

‡ d.i.—Data insufficient; usually blood samples were not taken early enough to evaluate rapid rate of equilibration.


0.25 gm NaSCN
0.25 gm NaSCN
S.K. 3/3/54
x—x % ADMINISTERED DOSE IN THYROID
$T_{\frac{1}{2}}$ = 9.7 min
$T_{\frac{1}{2}}$ = 9.0 min
TIME IN MINUTES FOLLOWING INTRAVENOUS ADMINISTRATION OF I^{131}

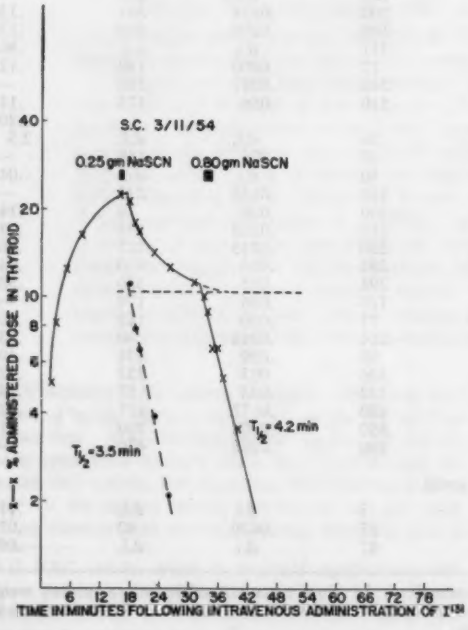

S.C. 3/11/54
0.25 gm NaSCN
0.80 gm NaSCN
x—x % ADMINISTERED DOSE IN THYROID
$T_{\frac{1}{2}}$ = 3.5 min
$T_{\frac{1}{2}}$ = 4.2 min
TIME IN MINUTES FOLLOWING INTRAVENOUS ADMINISTRATION OF I^{131}

FIG. 5. THE EFFECT OF GRADED DOSES OF THIOCYANATE ON THE RATE OF DISCHARGE OF TRAPPED IODIDE—BOTH SUBJECTS HYPERTHYROID

The x's are observed thyroidal radioactivities. The discontinuous horizontal lines represent the asymptotes to which the curves were apparently approaching after the first thiocyanate injection. The solid circles are the points of difference between the observed activities and the asymptotes.

In S. K., 0.25 gm. NaSCN discharged about 75 per cent of exchangeable thyroidal iodide. A repeat injection of the same dose of NaSCN discharged the remaining iodide at the same rate.

In S. C., 0.25 gm. NaSCN discharged about 50 per cent of thyroidal iodide. The remaining 50 per cent was discharged at the same rate by a repeat injection of 0.8 gm. NaSCN.

iodine (B) can be constructed (Figure 4). Then from (*9*),

$$\lambda_{TP} = \frac{dB/dt}{T} \qquad (12)$$

Estimates of the rate of binding of trapped iodide (λ_{TB}) can then be made from the slope of the bound iodine curve (B) and the value for trapped iodide (T) at the same time. To decrease the magnitude of the experimental errors, the ratio, $\frac{dB/dt}{T}$, may be obtained at a number of different time points on the same set of curves and the results averaged (Figure 4).

From the plasma concentration curve (P) and the trapped iodide curve (T), the thyroid/plasma ratio can be obtained at any time and compared with the ratio observed when binding is prevented by Tapazole®.

RESULTS

Thyroidal Trapping of Iodide; Binding Inhibited by Tapazole®

Values for T_s, λ_{TP} and "equilibrating thyroidal plasma flow" ($\lambda_{PT}V$) are given in Table I. In each case λ_{TP} was evaluated from several points on the curves, as in Figure 3, and an average taken of the several determinations.

Following equilibration with plasma iodide^{131}, trapped thyroidal iodide^{131} was discharged by the intravenous administration of 1.0 gram NaSCN given over a 1½ to 2 minute period.[7] The disappearance of radioactivity from the thyroid under these conditions was almost always exponential with half times varying from 0.25 to 23 minutes (Figures 2, 3a, 5, Table I). The rate of discharge was always greater than that given by λ_{TP} (Table I). In several cases an attempt was made to slow down the rate of discharge by the administration of smaller doses of NaSCN. However, the effect appeared to be simply a reduction in the fraction discharged; the rate of discharge of the radioactivity which disappeared remained unchanged (Figure 5).

Thyroidal Iodide Binding

The rate of binding of trapped iodide was evaluated from the results of three different types of experiments performed in the absence of Tapazole® block:

1) Determination of the thyroidal clearance rates of plasma iodide at different intervals during the half-hour period following administration of iodide^{131};
2) Determination of the dischargeability of radioactive iodine from the thyroid by the intravenous injection of NaSCN;
3) Determination of thyroidal iodide and the rate of binding according to the analysis presented above.

1. *Constancy of thyroidal clearance of plasma iodide*

When tracer iodide is administered in the absence of Tapazole® block, thyroidal plasma iodide clearance rates remain relatively constant (Figures 3, 6). These observations confirm previous studies (*7*). It must be emphasized that in extremely active glands these measurements may be valid only for the first 15 to 20 minutes since by this time the thyroid may already be secreting some organically bound I^{131} as evidenced by the appearance of significant concentrations of PBI^{131} (compared to iodide^{131}) in the plasma.

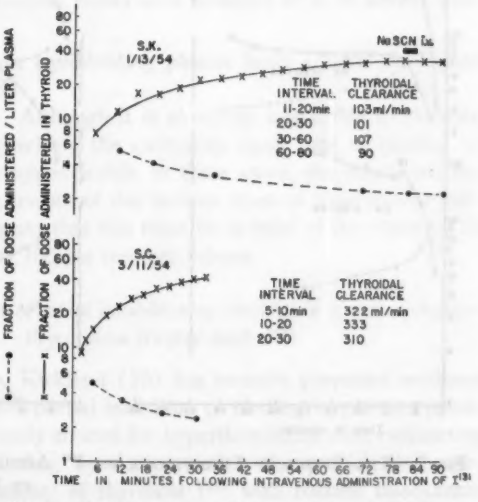


FIG. 6. THYROIDAL AND PLASMA RADIOACTIVITIES IN S. K. AND S. C. DURING "BINDING" STUDIES

Both subjects hyperthyroid. Thyroidal clearances remain constant throughout period of observations. See also H. W., Figure 3a.

[7] Although this dose of thiocyanate appeared to discharge all trapped iodide^{131}, it is possible that a low thyroid/plasma ratio of 2 or 3 to 1 persisted. Since this would generally mean a thyroidal iodide space of only about 0.1 liter, the persisting thyroidal radioactivity after NaSCN would represent only a fraction of a per cent of the administered dose and would not be detectable by the methods used in this study.

If there were an appreciable lag in binding of trapped iodide, iodine accumulation by the thyroid, binding permitted, would be a two-rate function. The clearance values should then decrease significantly with time. Since no detectable decrease was observed in these cases, the binding of trapped iodide was effected without significant time lag.

2. *Effect of NaSCN on thyroidal radioactivity in the absence of Tapazole® inhibition*

That binding is, in fact, extremely rapid compared to trapping was repeatedly confirmed by the failure of NaSCN to discharge any thyroidal activity which had been accumulated more than one minute prior to the administration of the NaSCN (Figure 7). In all cases (17 subjects) where NaSCN was injected between 9 and 50 minutes after the I^{131} tracer, no detectable decrease in thyroidal radioactivity was observed. D. H. is a possible exception. In two cases where thiocyanate was given at 4½ and 7 minutes (S. R. and J. J., respectively, Figure 7), there was evidence of discharge of some of the iodide accumulated during the period of thiocyanate injection, but not of any of the iodide accumulated prior to injection. The effectiveness of NaSCN, in these doses, in rapidly discharging trapped iodide when it is present, is evident from the experiments in the same subjects and in others blocked with Tapazole® (Figure 2).

The longevity of action of thiocyanate should be noted. In a number of cases a binding study was performed about a week after a trapping study in which thiocyanate had been used to discharge the iodide. In many of these cases the thyroidal radioactivities at early times during the binding study were actually lower than at corresponding times during the trapping study, apparently due to the inhibitory effect on iodide trapping by the

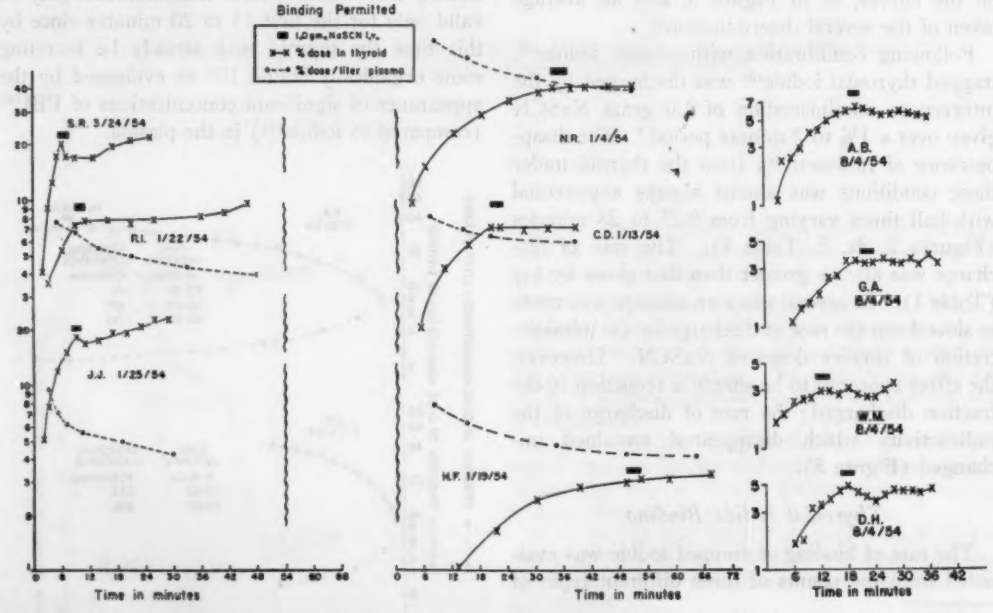


FIG. 7. THE EFFECT OF THIOCYANATE ON I^{131} ACCUMULATED BY THE THYROID IN THE ABSENCE OF INHIBITION OF BINDING

First five subjects (S. R. to C. D.) hyperthyroid; last five subjects (H. F. to D. H.) euthyroid. In N. R. the plasma points should be read as 0.1 × scale reading. The effect of NaSCN in discharging iodide^{131} when present may be observed in Figure 2.

Because of the low uptakes in the euthyroid subjects there was a larger statistical error in the counting rates which accounts for the waviness of the plateau regions after thiocyanate in these cases.

residual thiocyanate (compare, with dates, C. D., R. I., and J. J., Figure 7 and Figure 2). In a few cases thiocyanate given during the binding study appeared to inhibit completely further I^{131} uptake for only a short time; after that the thyroidal uptake slowly increased (S. R., R. I., and J. J., Figure 7). Apparently the fall from peak thiocyanate concentration due to distribution into slower equilibrating extrathyroidal spaces was sufficient to allow escape of the thyroid trap from complete thiocyanate inhibition in these cases.

3. *Determination of the rate of binding according to the kinetic analysis*

To attempt a more precise evaluation of the rate of binding of trapped iodide, trapping and binding studies were performed on the same day in several patients, as described under Methods, and analyzed as in Figure 3. Since less than two hours elapsed between the two studies in these cases, the possibility of significant changes in blood flow, transfer rate constants, mean iodide diffusion paths, *etc.* can be disregarded and the results may be directly compared. Several sets of the curves obtained are shown in Figure 8. As expected, the initial slopes are virtually identical since at 0 time there is only a one way passage of I^{131} (into the gland) whether or not binding occurs. Since the rate at which iodide^{131} leaves the gland during the trapping studies (λ_{TP}) is generally only 2 to 4 per cent per minute (Table I), it is not surprising also that the initial segments of the curves should appear to be virtually superimposable. In six previously untreated hyperthyroid patients, the analysis demonstrated that, when binding was uninhibited, no detectable amounts of iodide^{131} were present in the gland at any time, at least after the first blood samples were withdrawn (as early as 3½ to 5 minutes). In none of these cases was it possible to construct any curve at all for thyroidal iodide (T), small positive or negative values (within the limits of the experimental errors) being obtained at all points. These observations indicated that binding must have occurred almost instantaneously and that the thyroid/plasma gradient for exchangeable iodide must have been very low. Further confirmation that binding was virtually instantaneous is afforded by the almost identical values for the plasma iodide clearance rates and

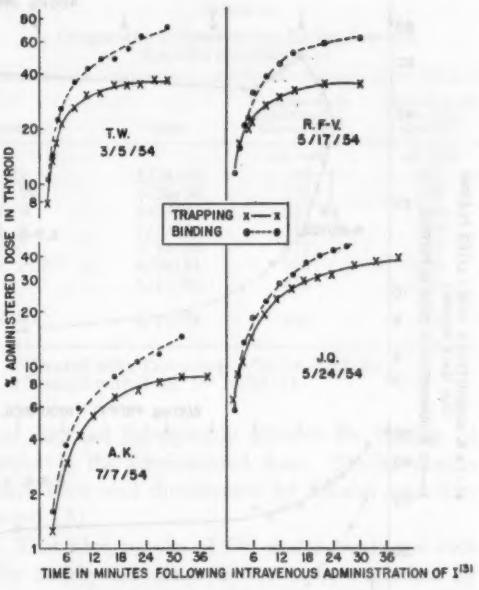


FIG. 8. THYROIDAL RADIOACTIVITIES (PER CENT ADMINISTERED DOSE) DURING TRAPPING AND BINDING STUDIES PERFORMED ON THE SAME DAY IN EACH OF FOUR HYPERTHYROID SUBJECTS

Fifty mg. Tapazole® was given to each subject a few minutes after the last point on the binding curves. The trapping studies were performed 60 to 90 minutes later.

the equilibrating plasma flows (Table II, Figure 3).

Although it is obviously impossible to evaluate precisely the extremely rapid rate of binding of trapped iodide in these cases, the combined experience of the various types of observations suggests that this must be at least of the order of 50 to 100 per cent per minute.

4. *Partial inhibition of binding in a case of hyperthyroidism treated with I^{131}*

Kirkland (10) has recently presented evidence for partial inhibition of binding in patients previously treated for hyperthyroidism with radioactive iodine, by demonstrating the partial dischargeability of thyroidal I^{131} with sodium thiocyanate several hours after the administration of radioiodine. This observation is confirmed by the tracer studies in subject R. S. (Figure 4), in whom a therapeutic dose of I^{131} five months previously

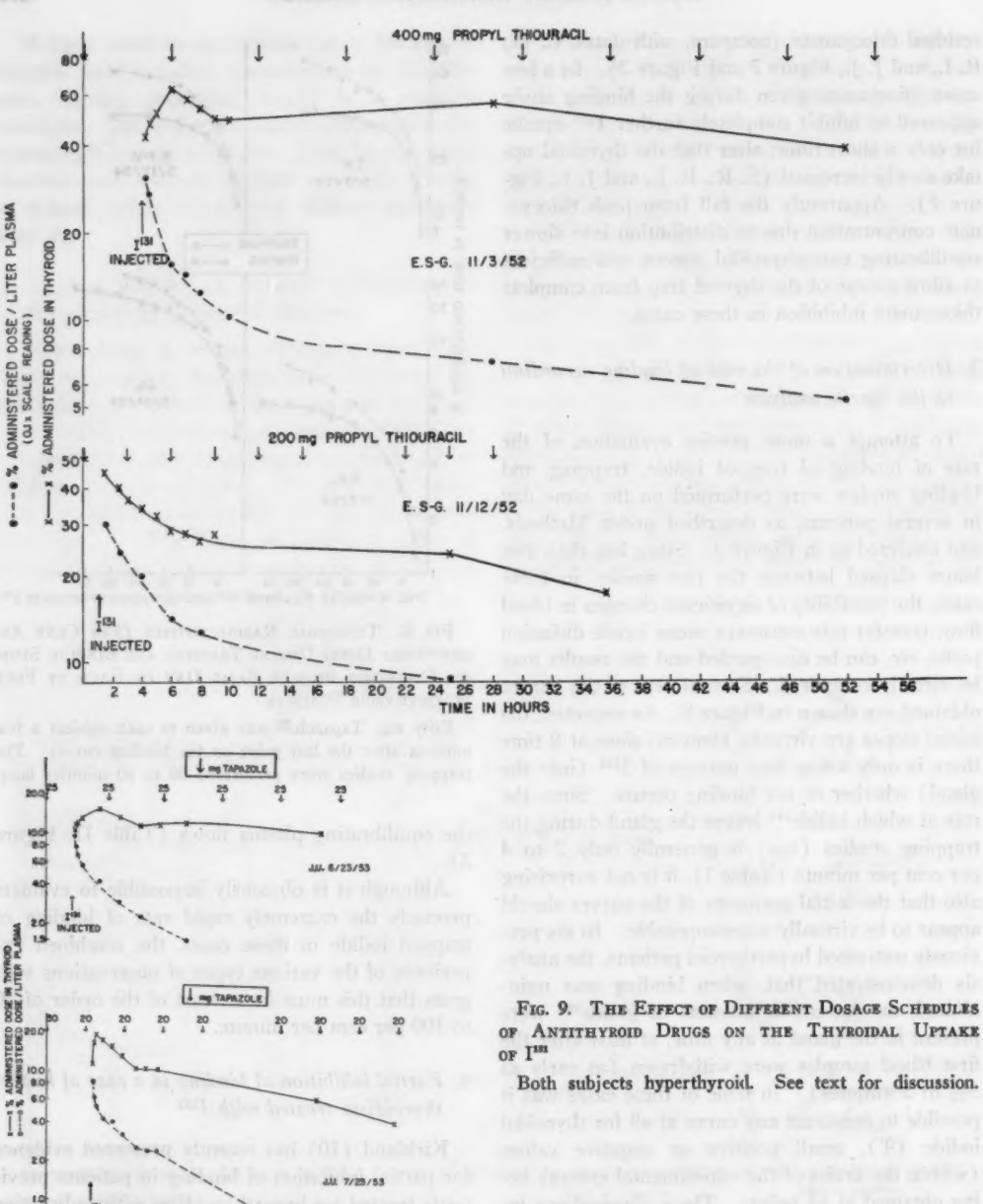


FIG. 9. THE EFFECT OF DIFFERENT DOSAGE SCHEDULES OF ANTITHYROID DRUGS ON THE THYROIDAL UPTAKE OF I^{131}

Both subjects hyperthyroid. See text for discussion.

had failed to produce complete remission of thyrotoxicity. In this case, the equilibrating plasma flow (143 ml. per min.) was distinctly higher than the observed thyroid clearance rate after 10 minutes (86 ml. per min.). Analysis of the trapping and binding curves (Figure 4) revealed that there was a detectable amount of trapped iodide present throughout the period of the binding study and that binding of the trapped iodide proceeded at a rate of about 10 per cent per min. The thyroidal iodide space (T_s) was 1.0 liters plasma equivalent as compared to 2.9 liters when binding was completely inhibited by Tapazole®.

TABLE II

Comparison of equilibrating plasma flow and thyroidal clearance rates

Subject	Date	Trapping study Equilibrating plasma flow	Binding study Thyroidal clearance
		ml./min.	*ml./min.*
J. Q.	5/24/54	390	347
H. W.	5/26/54	634	650
T. W.	3/5/54	553	540
A. K.	7/7/54	87	85
V. C.	3/16/54	177	167
R. F-V.*	4/26/54	700	800
	5/17/54	430	456
R. S.†	3/10/54	143	86

* Treated with Tapazole® 4/26/54–5/13/54.
† Treated with 8 mc. I^{131} 10/15/53.

Effect of Incomplete or Intermittent Inhibition of Binding by Antithyroid Drugs on the Thyroidal Uptake of Radioiodine

At completely inhibitory dose levels of antithyroid drugs, only a small fraction of the administered radioactive iodine remains in the thyroid (as unbound iodide) after 24 hours or so. For Tapazole®, a dosage schedule of 25 to 30 mg. every six hours has been found effective in reducing binding of radioactive iodine to less than 2 to 3 per cent of the administered dose in almost all cases. With smaller doses or longer intervals between doses a variable degree of binding is permitted and significant fractions of I^{131} are found in the thyroid 24 to 48 hours after administration of the tracer. In Table III are given the 24-hour thyroidal I^{131} uptake values for a group of hyperthyroid subjects maintained for one to two weeks on different dosage schedules. In all cases the total daily dose was divided into four equal parts given at 6-hour intervals. The 24-hour values observed are actually slightly in excess of the amounts bound in organic form, since a small fraction of thyroidal radioactivity at 24 hours (usually less than 3 to 4 per cent of the administered dose) is still unbound iodide. Furthermore, the results are not always directly comparable since in any individual test the fraction bound depends on the time of administration of the tracer with respect to the previous dose of antithyroid agent. However, the results demonstrate that in general the fraction of administered iodide^{131} which is bound decreases as the dose level is increased. Ten mg. of Tapazole® per day appeared to be almost totally ineffective and even 40 mg. per day not infrequently allowed for binding of about ⅓ the administered dose. Similar results have been well documented by Stanley and Astwood (3).

That high uptake of I^{131} under treatment with the antithyroid drugs is not to be explained by intrinsic thyroidal refractoriness to these agents is clear from the effect of increasing dose levels in the same subjects (Table II), and is further demonstrated by the two cases shown in Figure 9. In subject S. G. 400 mg. propylthiouracil every 6 hours permitted uptake of over 50 per cent of the tracer at 24 hours. When the same total daily dosage was divided into amounts of 200 mg. every 3 hours, inhibition of binding was much more effective, only 20 per cent remaining at 24 hours. It is to be noted that in the first test, the radioiodine was administered 5 hours after the last dose of propylthiouracil so that a large fraction of the I^{131} was bound before the next dose of propylthiouracil took effect. Apparently the effective duration of action of propylthiouracil in this case was between 3 and 5 hours. In other cases of hyperthyroidism, 200 mg. propylthiouracil every 3 hours has been effective in inhibiting almost completely the binding of iodide^{131}. In case J. U., complete inhibition of binding was not effected by 25 mg. of Tapazole® every 6 hours and 10 per cent of the dose was retained in the thyroid at 24 hours. By administering an extra 50 mg. before the test and 20 mg. every 4 hours during the day, the amount retained at 24 hours was reduced to 4 per cent (Figure 7). As suggested by the thyroid and blood curves, about 3 per cent of the ad-

TABLE III

Effect of varying daily dosages of Tapazole® on the 24-hour uptake of I^{131} (per cent of dose) *

Subject	mg./day.... 0	10	15	20	30	40	60
R. T.	63			27		30	
L. Fa.	75, 84			46	46	35	
P. S.	83	59, 65		25	25		
W. W.	84				37	23	
E. McI.	95, 77, 82			37, 35			
C. T.	84				50		
P. R.	79				28		
H. B.	74			24			
V. S.	83, 79, 78	65, 73		53	34, 26		
T. B.	80				27		
A. C.	90, 83				36, 28		
J. Lo.	83			72		44	
L. Fe.	71, 72				24, 22		
J. J.	77					28	
E. K.	75			26			
L. D. M.	47, 44		15, 26		16		
M. G.	78				20, 21		
J. Lu.	90						17
A. I.	77				31, 34		
J. B.	61				19		
A. F.	84, 91				13		
J. D.	82	85		35, 55	28, 26, 28, 29		

* Total daily dose divided into four equal quantities at 6-hour intervals. I^{131} tracer tests performed without discontinuing therapy after 1 to 2 weeks on the dosage schedule indicated. The 0 column indicates the values obtained prior to therapy or during a therapy-free interval.

ministered I^{131} was apparently bound during the night of the second test when a dose of Tapazole® was omitted. This was the only case observed in the present series in which 25 mg. Tapazole® every 6 hours permitted a detectable quantity of I^{131} to be bound.

DISCUSSION

Rate of Thyroidal Trapping of Iodide

The significant parameter for iodide trapping appears to be what has been here termed the equilibrating thyroidal plasma flow, in effect, an hypothetical volume flow from which the thyroid trap extracts all the iodide. When organic binding is completely blocked, an equal amount of iodide is returned to the plasma (at equilibrium) but in the absence of inhibition of binding this amount of iodide is quantitatively converted to organic form. It cannot be denied that some minute fraction of trapped iodide may be returned to the plasma without being bound in the thyroid but the findings presented in this study indicate that it is generally undetectable. There are special circumstances, however, where binding is not so efficient. Kirkland's report on inhibition of binding in glands previously subjected to significant radiation from I^{131} has already been cited and a case illustrating this situation has been presented. Iodide and the antithyroid drugs also, of course, inhibit binding. In addition to these examples of induced inhibition of binding there are instances of spontaneous physiologic or pathologic block to binding, such as the inability of the 7-day chick embryo thyroid to bind the iodide it is capable of trapping (11) and the limited binding of iodide trapped by the thyroids of certain goitrous cretins (12, 13). These cases must be distinguished, however, from the usual euthyroid or hyperthyroid subject whose gland appears to be capable of binding virtually all the iodide which is trapped before it can escape back to the plasma. In these cases, then, it is the rate of trapping rather than the rate of binding which is the limiting step in thyroidal iodine accumulation.

In discussing the trapping rate process it may be questioned whether it is not more pertinent to evaluate the quantity of I^{127} trapped per unit time than to consider only I^{131} transfer. However, experiments have been performed with carrier doses of iodide^{127} several hundred times the exchangeable iodide^{127} present in the body without affecting the equilibrating plasma flow rates or the T_s val-

ues (14).[8] Under these conditions the hyperplastic thyroid is capable of trapping several hundred micrograms iodide^{127} per minute. Thus, the thyroid trap is usually operating far below its capacity for iodide^{127} at transfer rates which are independent of the serum iodide^{127} concentrations within wide limits of the latter.

Thyroidal clearance of plasma iodide and the roles of trapping and binding

Myant, Pochin, and Goldie (15) and Berson, Yalow, Sorrentino, and Roswit (7) noted that the thyroidal clearance of plasma iodide^{131} remained fairly constant over periods of several hours after the administration of I^{131}. This constancy was observed even when clearance rates during the first and second 15-minute periods were compared with those from ½ to 3 hours following intravenous injection of radioiodine (7). It was, therefore, tacitly assumed that thyroidal clearance of iodide was a single rate function. The same assumption is implicit in the studies of Stanley (16) and of Keating and Albert (17). It has furthermore been assumed in these studies that the product of the rate of thyroidal plasma iodide^{131} clearance and the plasma iodide^{127} concentration may be considered quantitatively equal to the rate of hormonal iodine synthesis in the absence of spontaneous or induced block of synthesis.

More recently, Ingbar (4) has stated that the rate of thyroidal clearance of plasma iodide as calculated by previous investigators does not indicate the rate of transfer of plasma iodide to hormonal iodine. He has calculated that the average rate of binding of trapped thyroidal iodide is only 1½ per cent per minute in euthyroid and 6½ per cent per minute in hyperthyroid subjects. However, if this were true a very large fraction of thyroidal radioactivity should be in the form of exchangeable unbound iodide for some time after injection of I^{131}. The inability of NaSCN to discharge any significant fraction of radioactivity as observed in the cases presented here and in the studies of VanderLaan (18) offers strong evidence against the correctness of Ingbar's calculations. Moreover, Ingbar's studies were based on a number of questionable assumptions. His analysis assumes that, in unblocked glands, the total counting rate over the neck two minutes after the intravenous injection of iodide^{131} can be taken to represent unbound thyroidal iodide^{131} without correction for extrathyroidal radioactivity. Employing this value, the thyroidal iodide space at that time is then calculated, not from the actual plasma concentration, but from the 0 time exponential extrapolation of the late plasma curve. It is further assumed that the thyroid/plasma ratio remains constant thereafter and that thyroidal iodide^{131} at any time therefore can be calculated from the exponential extrapolation of the late plasma curve. These assumptions are unjustified and can be shown to be invalid.

The theoretical analysis presented by Wollman (6) requires that the plasma iodide^{131} concentration remain constant or decrease at a single exponential rate. Since the plasma concentration does not (and, theoretically, cannot) decrease exponentially during equilibration with the thyroid trap, the formulas derived cannot be directly applied to the precise analysis of data obtained following a single injection of I^{131}. The possibility of approaching the problem through the maintenance of constant plasma concentrations by continuous infusion techniques remains to be explored but would appear to be difficult since removal from the plasma is not constant. However, the method of analysis developed in the present report appears to satisfactorily resolve the difficulty of changing plasma concentrations.

Wollman's theoretical analysis can be recommended for its evaluation of the consequences of non-instantaneous equilibration of the thyroid trap. However, he concludes that "if equilibration cannot be considered instantaneous . . . the thyroid clearance of radioiodide from the blood will not be a constant independent of the time after injection." The observations presented here demonstrate that, in untreated glands, thyroid clearance is a constant for all practical purposes, because of the extremely rapid rate of binding.

Possible factors in the limitation of iodide trapping

To our knowledge, there have not been reported any data relative to the iodine extraction ratio in human hyperplastic thyroid glands. However,

[8] These observations refer only to acute experiments. Physiologic adjustments to continued treatment with iodide are not under consideration here.

since thyroidal plasma iodide^{131} clearance rates of 1200 ml. per min. have been observed in this laboratory, and even higher values have been reported by Myant, Pochin, and Goldie (15), it appears highly improbable that extraction is much less than completely efficient in these cases, unless the blood flow to the thyroid is to equal or exceed that to the rest of the body. In such cases then, it is suggested that iodide trapping is, at least in great part, limited by the blood flow. In the less active gland, however, the importance of other factors in limiting the rate of iodide trapping may be inferred from the following considerations.

The mean normal thyroidal plasma iodide^{131} clearance rate is approximately 17 ml. per min. (7, 15). From values for iodide^{127} excretion, renal clearance and daily hormonal requirements, Riggs arrives at a fairly similar estimate of 18.8 ml. per min. for the thyroidal clearance of iodide (19). Means (20) has estimated the normal thyroid mass at 20 gm. and blood flow at 4 ml. per gm. per min., implying a total thyroidal blood flow of 80 ml. per min. Using these estimates and assuming that RBC iodide is directly available to the thyroid, the values for thyroidal plasma clearance (employing an hematocrit of 45 per cent) indicate an extraction ratio of only 27 per cent in the normal gland. These figures are in good agreement with Pochin's direct observations on the normal lobe during surgical removal of nontoxic adenomata which gave a mean extraction ratio of 20 per cent (21).

Thus, it would appear that other factors, in addition to blood flow, probably contribute to the limitation of iodide trapping in the normal gland. The factors to be considered are those concerned with passage of iodide from the blood stream to the thyroid trap as well as possible limitations of the trapping mechanism for stable iodide. The possibility that overloading of the trapping mechanism is responsible for limiting the rate of iodide trapping in these cases has been rendered very unlikely by the experiments previously cited with large doses of iodide^{127} in which it was observed that in the same gland, amounts of iodide^{127} extending throughout ranges of 100-fold and greater are trapped at identical equilibrating plasma flow rates and thyroid/plasma ratios (14).

Analysis of the passage of iodide from the capillary to the trap cannot be reduced to simple terms. Schmidt (22, 23) has mathematically formulated the relevant factors involved in the rate of equilibration of molecules diffusing from blood to tissue, and Cotlove (24) recently has presented an elegant analysis of a number of special cases of extracellularly distributed substances. The factors involved, in addition to those concerned with blood flow, include the total capillary wall area, the permeability coefficient of the capillary wall to the particular molecule, the diffusion path length (here, the distance from the capillary to the trapping site) and the extent of reduction of the free diffusion coefficient due to geometrical and other peculiarities of the tissue (*e.g.*, the increase in diffusion path length over the straight line distance, necessitated by passage around fibers, *etc.*). Unfortunately there are no data for the thyroid available for any of these factors. Pitt-Rivers and Trotter (25) have presented evidence that in the thiouracil blocked gland the entire colloid area is the site of trapped iodide. Measurements of the straight line path between the capillary and the trapping site could be made without difficulty, and significant differences in follicular diameter of normal and hyperplastic glands might then be seen to be influential. However, there is no information available concerning the degree of hindrance of the colloid to the diffusion of iodide. Whether the diffusion coefficient of iodide in the colloid is reduced significantly below the free diffusion coefficient may be critical to the calculations and should be answered by future *in vitro* studies. The report by De Robertis (26) that the viscosity of the colloid diminishes after the injection of thyrotropic extracts of the pituitary may be pertinent in this regard. Whether the permeability coefficient of the thyroidal capillaries for iodide is a constant at varying levels of thyroidal activity is also unknown. Furthermore, in addition to the parameters concerned with equilibration in the extracellular tissues of the thyroid, there is the factor of iodide passage through or between the follicle cells to the colloid. The permeability of the follicle cells to iodide may be an important regulatory factor in the trapping of iodide. The present state of knowledge of these relevant physiologic problems does not permit even a guess as to the critical factors involved in the limitation of iodide trapping. Perhaps the

questions raised may point the way to future avenues of investigation.

Non-Exchangeable Thyroidal Iodide

Riggs (19) has estimated the average plasma iodide level of persons living in New England at approximately 3 micrograms per liter. Estimations of the plasma iodide concentrations in several patients of the present study (E. G., S. K., N. R.) have been given elsewhere (27), and were observed to be of the same order of magnitude. In these hyperthyroid subjects under the effects of Tapazole® block, exchangeable thyroidal iodide never exceeded 36 liters plasma equivalent or 108 micrograms iodide; in most cases, values of 10 micrograms or less would appear to represent the amount of exchangeable iodide present in the thyroid when binding is blocked. In unblocked normal or hyperfunctioning glands, moreover, the quantity of exchangeable thyroidal iodide must be significantly less. Yet much greater amounts of iodide have been found in the untreated thyroid on direct chemical analysis. Gutman, Benedict, Baxter, and Palmer (28) reported an average value of 580 micrograms for inorganic iodide on human thyroid glands obtained from apparently normal individuals who had met with sudden death. Braasch, Albert, Keating, and Black (29) have observed that approximately 6 to 7 per cent of total thyroidal I^{127} and I^{131} is identifiable as iodide on column chromatography of trypsin-hydrolyzed material or that approximately 1,000 micrograms of iodide are present as a maximum in the untreated thyroid gland of euthyroid and hyperthyroid subjects. Utilizing the techniques of filter paper partition chromatography on saline and trichloracetic acid extracts of rat, rabbit, sheep, and dog thyroids, Taurog, Tong, and Chaikoff (30) found that inorganic iodide constituted only about 1 per cent of total thyroidal iodine in these animals. However, even this latter value represents a very much larger quantity of thyroidal iodide than is derived by estimations from the equilibration studies with radioactive iodine. Therefore, if the finding of these large amounts of inorganic iodide on chemical analysis cannot be attributed to breakdown of organic iodine in the separation procedures utilized or to other artifacts, it must be concluded that the major portion of thyroidal iodide is not readily exchangeable with the iodide of the plasma. It is interesting to speculate whether this unexchangeable iodide exists in the colloid side by side with exchangeable iodide or resides only in adjacent inactive follicles.

The Kinetics of Iodide Discharge by Thiocyanate

It is likely that the mechanism of action of thiocyanate in discharging trapped thyroidal iodide will not be defined precisely until more is known about the fundamental nature of the iodide trap itself. However, several interesting observations of the kinetics of iodide discharge by thiocyanate merit consideration.

A priori, the possibility of three general hypothetical mechanisms for the action of thiocyanate may be postulated:

1. Thiocyanate prevents further entry of iodide into the trap but does not actively discharge the trap.
2. Thiocyanate actively discharges the trap.
3. Thiocyanate neither prevents iodide trapping nor discharges the trap but merely competes with iodide within the trap.

Either or both of the first two actions could be effected through an iodide trapping enzyme system if such exists.

The rapid rate of discharge of iodide by thiocyanate serves to exclude the first hypothesis. For, if thiocyanate merely prevented further iodide trapping, the rate of discharge by thiocyanate should have been identical to the transfer rate of iodide from thyroid to blood (λ_{TP}) determined during the equilibration period. In all cases the rate of discharge by SCN^- was greater than λ_{TP} and, in some instances, ten or more times as great.

The third mechanism has been suggested by Baumann and Metzger (31) on the basis of the similarity of behaviour of iodine and thiocyanate in the Hoffmeister series and other chemical likenesses. It has been stated that iodide concentration by the thyroid is not unique and that other halides and thiocyanate are also concentrated (31). Although it has been reported by others (32) that there is no thyroid/plasma concentration gradient for thiocyanate ion, this in itself does not exclude the competition theory. The administration of approximately 500,000 to 1,000,000 micrograms of

thiocyanate is generally required to discharge all the trapped iodide. On the basis of a space of distribution of 25 per cent of body weight in a 70 kg. man, one gram of NaSCN would give a concentration of 70 micromoles per cent in the plasma. If the same concentration were achieved in a 100 gram thyroid, SCN^- ion could simply, by substitution mole for mole, wash out almost 99 per cent of the less than 1.0 micromole of exchangeable iodide usually present in the blocked gland. Moreover, it is conceivable that one mole of thiocyanate could substitute for a number of moles of iodide. The exponential nature of the discharge curve, which is suggestive of an exchange or turnover reaction, and the unchanging rate of discharge when different amounts of thiocyanate were used to release different fractions of thyroidal iodide, are consistent with the competition hypothesis. However, certain experimental observations fail to add support. The same dose of thiocyanate is capable of discharging, entirely, milligram amounts of iodide from thyroids equilibrated with large carrier doses of I^{127} (14), which is not consonant with the concept that the graded amounts of thiocyanate were required to discharge by replacement only a few additional micrograms. This observation suggests that thiocyanate acts in a quantitative manner on some part of the iodide trapping mechanism which is independent of the amount of iodide trapped. For example, an individual locus of trapping may be able to hold a variable number of iodide ions. The presence of a single thiocyanate radicle however might exclude any iodide ions from that locus. This should not be interpreted as a substitution phenomenon but rather as an active discharge of the trapped iodide or a poisoning (albeit temporary) of the iodide trapping site.

The experimental observations and foregoing speculations therefore suggest that sodium thiocyanate actively discharges iodide from the thyroid. If so, it seems difficult to interpret the exponential character of the iodide discharge curve in any other way than by postulating a sudden release of iodide from the thyroid trap followed by a "wash out" of the iodide. In some hyperthyroid cases, at least, the rate of "wash out" was too slow to be limited by blood flow since in many of these cases flows of 500 ml. per min. or more were perfusing glands of only 50 to 100 grams. If diffusion equilibrium within the gland were instantaneous, the half times for discharge should then have been only of the order of seconds, rather than minutes as observed. The discharge of iodide by thiocyanate, therefore, is, in all probability, chiefly diffusion and/or permeability limited which suggests that these factors play some role in the accumulation process as well.

SUMMARY AND CONCLUSIONS

1. A method is presented for the kinetic analysis of thyroidal iodide trapping and binding *in vivo*.

2. Radioiodide transfer between thyroid and plasma in both directions was determined in a group of subjects under conditions in which organic iodine binding was effectively blocked.

3. In the absence of block of iodide binding, thyroidal plasma clearance of $iodide^{131}$ is essentially constant and the thyroid/plasma $iodide^{131}$ ratio is too small to be measured (by the *in vivo* techniques employed here) in hyperthyroid as well as euthyroid subjects.

4. Thyroidal plasma iodide clearance in the unblocked gland is equal to that volume flow of plasma whose iodide content is transferred to the thyroid trap in the blocked gland. This indicates that iodide trapped by the thyroid is bound before significant loss back to the plasma can occur. Further confirmation of the efficiency and rapidity of binding of trapped iodide by the untreated gland is presented in experiments demonstrating the absence of discharge, by thiocyanate, of any significant fraction of accumulated thyroidal radioactivity even a few minutes after the injection of radioactive iodine.

5. Factors possibly limiting thyroidal iodide trapping have been discussed in terms of blood flow, permeability and diffusion and are considered inadequately defined in the quantitative aspects at present to justify conclusions regarding their relative significance.

6. Possible mechanisms of thiocyanate discharge of trapped thyroidal iodide have been considered. The quantitative experimental data presented favor the interpretation of an inactivation of the thyroid trap by thiocyanate in preference to that of a simple substitution of thiocyanate for iodide.

7. The effect of different dosage regimens of l-methyl-2 mercaptoimidazole on iodide binding and on the character of the thyroidal I^{131} uptake curves is presented.

APPENDIX

Alternative Analysis of Iodide Trapping

Let T and P be total thyroidal iodide[131] and iodide[131] concentration in the plasma, respectively, and T* and P* be total exchangeable thyroidal iodide[127] and iodide[127] concentration in plasma, respectively, at any time, t. Also, let $T_s^* = \frac{T^*}{P^*}$ and V be the plasma volume. Then,

$$\frac{dT}{dt} = -\lambda_{TP}T + \lambda_{PT}PV \qquad (1)$$

In the steady state,

$$\lambda_{TP}T^* = \lambda_{PT}P^*V$$

and

$$\lambda_{TP}T_s^* = \lambda_{PT}V \qquad (2)$$

Substituting for $\lambda_{PT}V$ in (*1*),

$$\frac{dT}{dt} = -\lambda_{TP}T + \lambda_{TP}T_s^*P \qquad (3)$$

Solving for λ_{TP},

$$\lambda_{TP} = \frac{dT/dt}{T_s^*P - T} \qquad (4)$$

At distribution equilibrium, $\frac{T}{P}$ is approximately equal to $\frac{T^*}{P^*}$ (see below), so that

$$\lambda_{TP} \cong \frac{dT/dt}{T_sP - T} \qquad (5)$$

where T_s is the equilibrium value for $\frac{T}{P}$.

Since there is continual loss of I^{131} from the body by renal excretion, the equilibrium thyroid/plasma ratio for I^{131} exceeds slightly that for I^{127} as follows: From (*1*)

$$\frac{dT/dt}{T} = -\lambda_{TP} + \lambda_{PT}V\frac{P}{T} \qquad (6)$$

After distribution equilibrium is attained the fractional rate of decrease of thyroidal I^{131}, $\left(\frac{dT/dt}{T}\right)$, is constant and equal to the fractional rate of loss of total body I^{131}. Let the rate constant of loss from the body after equilibrium be K, and substituting in (*6*), $K = -\lambda_{TP} + \lambda_{PT}V\frac{P}{T}$. Then, $T_s(K + \lambda_{TP}) = \lambda_{PT}V$, and from (*2*),

$$\frac{T_s}{T_s^*} = \frac{\lambda_{TP}}{\lambda_{TP} + K} \qquad (7)$$

The rate constant of excretion after distribution equilibrium (K), in the hyperthyroid subjects studied, ranged from 0.0005 min.$^{-1}$ to 0.0010 min.$^{-1}$ (3 to 6 per cent per hr.) while the values for λ_{TP} fell between 0.0155 min.$^{-1}$ and 0.099 min.$^{-1}$ (see Table I). Thus, it is seen from equation 7 that T_s exceeds T_s^* (K is a negative quantity) by an average of about 1 per cent and never more than by about 6 per cent. By substituting in (*7*) the values obtained for λ_{TP}, K and T_s in any particular case, T_s^* may be determined almost precisely. By substituting this value for T_s^* in equation (*4*), λ_{TP} may now be recalculated, to yield a value into which an error of less than 1 per cent is introduced by the theoretical analysis.

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NEUTRALIZATION OF INFUSED ACID BY NEPHRECTOMIZED DOGS[1]

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From 8 to 15 mM of strong mineral acid per kilogram of body weight may be infused intravenously in the dog over a period of a few hours to produce a severe but non-fatal acidosis. Van Slyke and Cullen (1) showed that only one-sixth of the infused acid is neutralized by blood buffers, five-sixths presumably being neutralized by bicarbonate in interstitial fluid and lymph and by intracellular phosphate and proteinate buffers. Recently, Bergstrom and Wallace(2) have indicated that sodium and potassium on the surface of bone crystals are also available for neutralization of acid.

How tissue buffers participate in the neutralization of acid has been investigated by measuring changes in total quantity of extracellular sodium, potassium, bicarbonate, and chloride ions following infusion of hydrochloric acid into nephrectomized dogs. Our preliminary reports (3, 4) were based on experiments in which extracellular fluid volume was approximated by inulin distribution. The observation of a considerable increase in the inulin volume of distribution following infusion of acid led us to compare volumes of distribution of several substances proposed by others as approximations of extracellular fluid volume. This comparison indicated that radiosulfate distribution more closely measures extracellular fluid volume than does inulin in the whole animal (5). Therefore, the experiments on the effects of hydrochloric acid infusion have been repeated using radiosulfate distribution to measure changes in extracellular fluid volume. No definitive publication of the original experiments is contemplated.

The results of our recent experiments indicate that when 10 mM of hydrochloric acid per kilogram of body weight are infused into a nephrectomized dog over a period of two hours, 43 per cent of the infused acid is neutralized by bicarbonate originally present in extracellular fluid, whereas 51 per cent is neutralized by a process in which hydrogen ions are exchanged for sodium and potassium ions which diffuse into extracellular fluid probably from cells. Simultaneously a 16 per cent increase in extracellular fluid volume occurs.

The results of our earlier experiments using inulin agree qualitatively with each of these findings. The results differ quantitatively for the volume of distribution of inulin is less than that of radiosulfate (5), and in our earlier experiments correction was not made for the slow disappearances of inulin after two to three hours of equilibration.

EXPERIMENTAL PLAN

Dogs were nephrectomized immediately before an experiment to eliminate renal excretion of acid and to measure more accurately the volume of distribution of radiosulfate. Control values for sodium, potassium, bicarbonate, and chloride in extracellular fluid and in the circulating erythrocyte mass were established three and four hours after nephrectomy. One and one-half and two and one-half hours after the production of a severe metabolic acidosis by hydrochloric acid infusion the total amounts of these ions in the extracellular fluid and in the erythrocyte mass were re-established. Ion shifts into or out of the extracellular fluid and circulating erythrocyte mass were calculated from the differences in mean ion contents observed in the two control and two acidosis observations.

Four control experiments were performed under conditions comparable except that in three acid was not infused, and in one only 3 per cent glucose was infused in a volume comparable to the volume of the acid infusions.

EXPERIMENTAL PROCEDURE

Healthy, moderately lean, adult male dogs were lightly anesthetized with sodium pentobarbital, weighed and nephrectomized bilaterally. Radiosulfate ($S^{35}O_4$) and (in two of five experiments) radiochloride (Cl^{36}) were infused as previously described (5). Two and one-half, three, three and one-half and four hours after infusion of radioisotopes arterial blood samples were drawn for measurement of plasma radiosulfate concentration and

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plasma specific gravity. Additional samples were obtained at three and four hours for plasma and whole blood sodium, potassium, CO_2, chloride and for plasma radiochloride and inorganic phosphate concentrations, plasma pH, whole blood water content and hematocrit. Following the four-hour blood sampling, 0.3 N HCl totalling approximately 10 mM per Kg. of body weight was infused simultaneously through each of two polyethylene catheters, one inserted through a femoral vein into the inferior vena cava, the other inserted through an external jugular vein into the superior vena cava. Acid was infused at a rate of 2 ml. per minute through each of the catheters.

One hour following completion of the infusion of acid and at half-hour intervals thereafter, four arterial blood samples were drawn for plasma radiosulfate concentration and plasma specific gravity. Additional samples were drawn at one and one-half and two and one-half hours following completion of the acid infusion for plasma and whole blood sodium, potassium, CO_2 and chloride and for plasma radiochloride and inorganic phosphate concentrations, plasma pH, whole blood water content and hematocrit. Plasma volume was measured by T-1824 dilution (6) one and one-half hours before the infusion of acid was started and two hours after the infusion was completed.

Approximately 120 ml. of blood were drawn before and a similar amount following acid infusion. Autopsy following each experiment verified that renal excretion had been completely eliminated and that hemorrhage at the operative sites had not occurred.

ANALYTICAL METHODS

Whole blood water content was measured by weighing 1 ml. samples and drying at 105° C. for 24 hours. Plasma water content, hematocrit, plasma radiosulfate and radiochloride concentrations were measured by methods previously described (5). These radioisotopes do not mutually interfere in these methods. Appropriate corrections for self-absorption for radiochloride were applied for changing plasma chloride concentrations. Plasma and whole blood sodium and potassium concentrations in heparinized samples were measured by internal standard flame photometry. Samples for plasma pH and plasma and whole blood CO_2 and chloride were collected anerobically in oiled, oxalated syringes and plasma fractions promptly separated. Plasma pH was measured at 37° C. with a water-jacketed Cambridge glass electrode, plasma and whole blood CO_2 by the method of Van Slyke and Neill (7) and plasma and whole blood chloride by the method of Van Slyke and Hiller (8). Whole blood chloride was measured in modified Folin-Wu filtrates (9). Plasma inorganic phosphate was measured by a modification of the method of Fiske and SubbaRow (10). All analyses were made in duplicate.

CALCULATIONS

1. Plasma concentrations have been corrected for plasma water content. Interstitial fluid concentrations

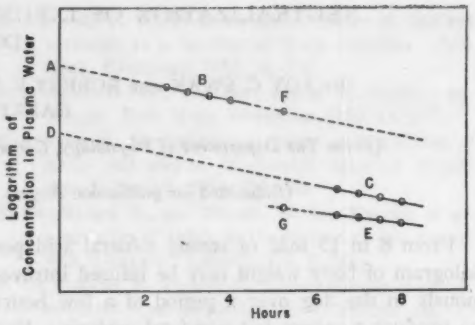

FIG. 1. CALCULATION OF INITIAL AND FINAL EXTRACELLULAR FLUID VOLUMES

have been calculated from plasma water concentration by applying a Donnan factor of 0.95 for all monovalent ions. Bicarbonate concentration has been calculated from CO_2 concentration and pH using the Henderson-Hasselbalch equation.

2. Total exchangeable chloride has been calculated by dividing total radiochloride activity injected by plasma specific activity. The radiosulfate volume prior to infusion has been calculated from zero-time concentration (A, Figure 1) obtained by extrapolation of a semilogarithmic plot using a formula (5) which includes correction for plasma volume and Donnan distribution. In all experiments the radiosulfate volume increased following infusion of hydrochloric acid. In two experiments (No. 2, No. 3) the slope of the disappearance curve (B, Figure 1) of radiosulfate remained unchanged after infusion, the curve merely being displaced to a lower range of concentrations (C, Figure 1). In these experiments the radiosulfate volume following infusion has been calculated from the concentration of radiosulfate in plasma water obtained by extrapolating to zero time (D, Figure 1) the disappearance curve obtained after infusion. In the other experiments (No. 1, No. 4, and No. 5) the slope of the disappearance curve (E, Figure 1) decreased slightly following infusion. The post-infusion volume has been calculated by assuming that the slope decreased progressively during and shortly after the infusion. The original curve (B) has been extrapolated to a point midway in time between the two curves. From this concentration (F) and the initial radiosulfate volume total radiosulfate activity in the radiosulfate volume at the midpoint has been calculated. The post-infusion volume has been calculated by extrapolating curve E to the midpoint (G). From this concentration and the total activity, the radiosulfate volume following infusion has been calculated.

3. Concentrations of water, sodium, potassium, and chloride in erythrocytes have been calculated from their plasma and whole blood concentrations and the hematocrit. Erythrocyte bicarbonate concentration has been calculated from the bicarbonate concentration in plasma water and a Donnan factor for chloride derived in each

experiment from the chloride concentrations in the water of plasma and erythrocytes. (This factor averaged 0.64 in control periods and 0.80 after acid infusion.) Total erythrocyte sodium, potassium, bicarbonate, and chloride have been calculated from their concentrations in erythrocytes and the circulating erythrocyte volume. Circulating erythrocyte volume has been calculated from measured plasma volume and hematocrit. Corrections have not been made for trapped plasma or difference in small and large vessel hematocrit.

RESULTS

Detailed data from a representative experiment are presented in Table I. The infusion of 161 mM of hydrochloric acid into a 16.8 Kg. dog reduced plasma pH from 7.41 to 7.10 and bicarbonate concentration in plasma water from 24.6 to 7.0 mM per L. Potassium concentration rose from 4.1 to 10.3 mM per L. The increment in total body chloride (158 mM) agrees closely with the amount of chloride infused (161 mM). As a result of the infusion of acid, there was an increase in total extracellular sodium of 55 mM and in potassium of 31 mM. Sixty-seven mEq. of base were derived from extracellular bicarbonate as CO_2 was eliminated. The increment in extracellular chloride approximates the amount of chloride given as hydrochloric acid despite the observed transfer of 18 mM of chloride into erythrocytes. No significant change in total sodium or potassium in circulating erythrocytes was observed. The rise in hematocrit is in part the result of increase in erythrocyte water concentration, but in large part probably due to contraction of the congested spleen of the barbitalized dog.

The results of the five experiments are summarized in Table II. The five dogs, averaging 18.9 Kg., received an average of 9.5 mM of hydrochloric acid per Kg. Extracellular fluid volume as measured by radiosulfate distribution, averaging initially 23.6 per cent of body weight, increased an average of 715 ml. An average of 615 ml. of acid was infused. Thus extracellular fluid volume increased by more than the volume of acid infused. Total sodium in extracellular fluid increased 65 mM, equivalent to 36 per cent of infused hydrochloric acid. Total potassium in extracellular fluid increased 28 mM, equivalent to 15 per cent of infused hydrochloric acid. Base derived from bicarbonate in extracellular fluid averaged 78 mM, equivalent to 43 per cent of infused hydrochloric acid. Total chloride in extracellular fluid increased 170 mM. Thus all but 6 per cent of the chloride infused as hydrochloric acid remained in the radiosulfate volume of distribution. Total exchangeable chloride as measured by radiochloride distribution increased by 98 per cent (Exp. No. 4) and 106 per cent (Exp. No. 5) of the chloride given.

In control experiments plasma volume decreased an average of 9 per cent. Plasma pH increased from an average of 7.40 to 7.43. Hematocrit increased an average of 7 per cent. Total exchange-

TABLE I

Average changes in total millimols of each monovalent ion in extracellular fluid and in circulating erythrocyte mass derived from detailed data from a representative experiment

Elaps. Time	Plasma Water Vol.	$S^{35}O_4$ Vol.	Hemat-ocrit	Plasma pH	Conc. in Plasma Water					Total Cl	Extracellular				Conc. in Erythrocytes				
					Na	K	HCO_3	Cl	PO_4	Cl	Na	K	HCO_3	Cl	Na	K	HCO_3	Cl	H_2O
hours	ml.	ml.			millimols per liter					mM	total millimols				millimols per liter				%
3.00	660	3990	.425	7.40	155.7	4.0	24.6	119.0	1.7	551	594	15	102	496	105	7.2	9.9	48.7	62.9
4.00			.415	7.42	156.9	4.1	24.5	119.0	1.6	548	597	15	102	496	104	7.7	10.0	47.5	63.7
4.16 – 6.50	Infusion of 550 ml. of 0.291 N HCl (161 mM), 16.8 Kg. Dog																		
7.75	675	4695	.467	7.11	145.1	10.3	6.7	134.3	1.9	706	652	46	34	658	94	8.0	3.4	68.9	65.4
8.45			.462	7.09	144.5	10.3	7.3	133.5	2.0	710	650	46	36	654	94	7.8	3.6	65.3	65.1
Average Change (total millimols)										+158	+55	+31	−67	+160	+5	+1	−4	+18	

TABLE II

Summary of data from the five experiments

EXP. No.	Wt.	HCl INFUSED			Δ TOTAL Cl	Δ TOTAL $^{35}SO_4$ VOL.	FINAL PLASMA pH	Δ CONC. IN PLASMA WATER				Δ TOTAL EXTRACELLULAR				%HCl NEUTRALIZED BY			
		TOTAL	mM./Kg.	VOL.												TRANSF. TO E.C.F.		HCO_3 IN E.C.F.	TRANS. OF Cl
	Kg.	mM.		ml.	mM.	ml.		Na	K	HCO_3	Cl	Na	K	HCO_3	Cl	Na	K		
								millimols per liter				millimols							
1	18.1	190	10.5	650		+765	7.09	-6	+4	-16	+14	+87	+25	-78	+173	46	13	41	9
2	19.0	169	8.9	560		+565	7.15	-10	+5	-18	+14	+38	+27	-80	+142	23	16	47	16
3	20.6	189	9.2	650		+755	7.05	-6	+4	-19	+19	+80	+21	-87	+194	42	11	46	-3
4	16.8	161	9.6	550	158	+705	7.10	-11	+6	-18	+15	+55	+31	-67	+160	34	19	42	1
5	20.2	194	9.6	665	204	+785	6.96	-9	+6	-16	+15	+67	+35	-78	+182	35	18	40	6
Av.	18.9	180	9.5	615		+715		-8	+5	-17	+15	+65	+28	-78	+170	36	15	43	6

able chloride remained constant. There was no significant change in the calculated total sodium, potassium, bicarbonate, and chloride in extracellular fluid.

Change in total sodium, potassium, bicarbonate, and chloride in circulating erythrocytes after acid infusion averaged + 3 mM (range for the five experiments, − 10 to + 23), + 1 mM (− 1.2 to + 2.6), − 4 (− 2.8 to − 6.2) and + 22 mM (+ 12 to + 29), respectively. In four control experiments change in total sodium, potassium, bicarbonate, and chloride in circulating erythrocytes averaged − 4 mM (− 24 to + 8), − 0.1 mM (− 0.9 to + 0.9), − 1.1 mM (− 2.3 to + 1.5) and − 1 mM (− 15 to + 6), respectively. Thus only for total erythrocyte bicarbonate and chloride are there significant differences between the control experiments and the experiments in which hydrochloric acid was infused. Total circulating erythrocyte bicarbonate decreased 4 mM. Total circulating erythrocyte chloride increased 22 mM, equivalent to 12 per cent of the infused chloride.

TABLE III

Change in osmolarity of plasma water due to monovalent ions and change in anion deficit of plasma water following infusion of acid

Exp. No.	milliosmols per liter (Na + K + HCO_3 + Cl)		Anion Deficit (mEq/L) (Na + K − HCO_3 − Cl)	
	Before HCl	After HCl	Before HCl	After HCl
1	300.8	297.3	16.3	18.2
2	308.9	299.2	17.4	17.3
3	300.1	297.0	20.4	17.8
4	303.9	296.0	16.8	14.2
5	303.5	297.9	15.1	14.3
Av.	303.4	297.5	17.2	16.4

Table III summarizes changes in osmolarity of plasma water contributed by the four monovalent ions, sodium, potassium, bicarbonate, and chloride, and changes in anion deficit (Na + K − Cl − HCO_3) as a result of infusion of acid. Osmolarity decreased 5.9 mOsm. per liter. The difference between monovalent cations (Na and K) and monovalent anions (HCO_3 and Cl) decreased 0.8 mEq. per liter, in part the resultant of an expected decrease in base binding of plasma proteins of 2 mEq. per liter as pH fell from 7.4 to 7.1 and an average increment in phosphate concentration of 0.5 mM per liter following infusion of acid.

In the three control experiments in which there was no infusion, osmolarity contributed by monovalent ions decreased an average of 2.5 mOsm. per liter, and the anion deficit increased 1.6 mEq. per liter.

DISCUSSION

In these experiments a large amount of strong acid containing a non-metabolizable, non-volatile anion whose distribution is largely extracellular has been infused over a short period of time. Under these conditions, 43 per cent of the infused acid is neutralized by extracellular bicarbonate as CO_2 is eliminated by hyperventilation. Fifty-one per cent is neutralized by exchange of hydrogen ions for sodium ions and, to a lesser extent, potassium ions which diffuse into extracellular fluid along with water. In agreement with Van Slyke and Cullen (1) only about 18 per cent of the infused acid is neutralized by circulating blood.

A. Validity of observed increase in extracellular fluid volume

The validity of the observed increase in extracellular fluid volume following infusion of acid is crucial in interpreting the results of these experiments. Data sufficient to calculate this increase are presented in Figure 2. In each experiment the disappearance curve for radiosulfate was displaced to a lower range of concentrations. This displacement is considerably greater than that expected from the infusion of water equal in volume to the acid infused.

The observed expansion of extracellular fluid might be an artefact: a) If plasma sulfate concentration increased in acidosis enough to significantly lower measured radioactivity through self-absorption; b) if in acidosis there is an increased permeability to radiosulfate of cell membranes or an increased diffusibility into extracellular fluid not included in the initial radiosulfate volume of distribution; or c) if edema fluid accumulating at operative sites after radiosulfate infusion but prior to acid infusion remains unequilibrated with plasma with the result that radiosulfate concentration in edema fluid exceeds that in well perfused areas.

The first source of error is eliminated by adding carrier sulfate in an amount one thousand times that in plasma samples taken for assaying radiosulfate activity. This is confirmed by the fact that recoveries of radiosulfate from plasma obtained after acid infusion are satisfactory. The second possible source of error is unlikely for the following reasons. Comparable increases in inulin volume of distribution (11) and in mannitol volume of distribution (12) have been observed after infusion of acid. The slope of the disappearance curve for radiosulfate remains unchanged or decreases slightly after acid infusion. In studies of respiratory acidosis and alkalosis under comparable experimental conditions, Giebisch, Berger, and Pitts (13) have observed no change in slope of the disappearance curve for radiosulfate as plasma pH varies from 6.8 to 7.8. The third possible source of error is unlikely for the following reasons. No consistent differences in concentrations of monovalent ions or in the specific activity of radiochloride are seen between the two plasma samples obtained after acid infusion. The plot of the logarithm of radiosulfate concentration remains essentially linear after infusion of acid. We believe the increase in radiosulfate volume after the infusion of acid closely approximates the change in extracellular fluid volume.

However, the calculated increment in extracellular chloride in some experiments equals the amount of chloride infused and for all experiments

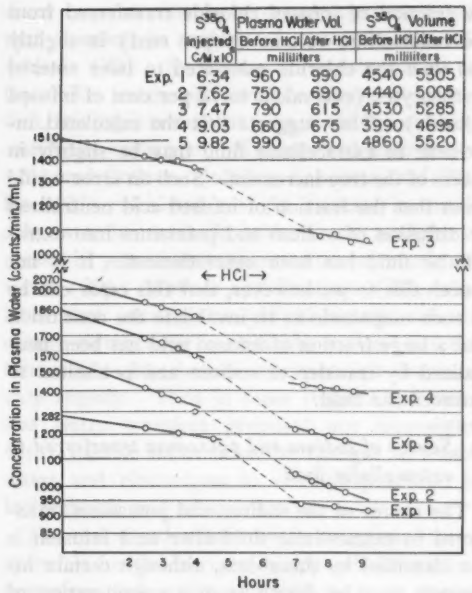

FIG. 2. SEMILOGARITHMIC PLOT OF RADIOSULFATE CONCENTRATION IN PLASMA WATER AGAINST TIME BEFORE AND AFTER INFUSION OF HYDROCHLORIC ACID

the fraction of infused chloride transferred from the radiosulfate volume (6 per cent) is slightly less than the chloride calculated to have entered erythrocytes (equivalent to 12 per cent of infused chloride). This suggests that the calculated increment in extracellular fluid may be slightly in excess of the true increment. Such an error would mean that the fraction of infused acid neutralized by diffusion of sodium and potassium into extracellular fluid has been overestimated. It is not conceivable to us, however, that this error can be of such magnitude as to invalidate the conclusion that a large fraction of infused acid has been neutralized by transfer of sodium and potassium to extracellular fluid.

B. Source of sodium and potassium transferred to extracellular fluid

The source of the sodium and potassium transferred to extracellular fluid after acid infusion is not identified by these data, although certain inferences may be drawn from a consideration of change in total osmolarity of plasma contributed by the major monovalent ions.

The results of these experiments indicate that a significant fraction of chloride does not enter cells other than erythrocytes following hydrochloric acid infusion. Were this to occur, intracellular osmotic pressure might be expected to increase, with transfer of water to cells and contraction of extracellular fluid. Such a transfer has been described for erythrocytes (14) and is apparent in these data on erythrocyte water and ion content.

With infused chloride largely confined to extracellular fluid, it is conceivable that intracellular buffers participate in the neutralization of infused acid through a process in which intracellular sodium and potassium exchange for hydrogen ion (11, 15, 16). The net effect would be the addition of sodium, potassium and chloride to extracellular fluid, a decrease in intracellular pH and a decrease in intracellular osmotic pressure due to the less ionized state of hydrogen, contrasted with sodium and potassium, in the presence of phosphate and proteinate. Calculations based on average values for the five experiments (Tables II and III), assuming an initial body water of 60 per cent of body weight and osmotic equilibrium between cells and extracellular fluid, support this possibility.

An 18.9 Kg. dog (Table II) has an initial body water of 11.4 liters, an initial radiosulfate volume of 4.47 liters (average of the five experiments) and an osmolarity of plasma water due to monovalent ions of 303.4 mOsm. per liter (Table III). One hundred and eighty mM (360 mOsm.) of hydrochloric acid in 615 ml. of water were infused into this average dog. Forty-three per cent of the infused hydrochloric acid is neutralized by extracellular bicarbonate. Acid thus neutralized does not effect a net increase in osmotically active solute since chloride merely replaces bicarbonate. Therefore, at most, only 205 mOsm. ($360 - [360 \times 0.43]$) have been added to body fluids.

Twenty-two mM of hydrochloric acid were apparently neutralized by erythrocyte buffers, approximately 4 mM by bicarbonate and presumably 18 mM by hemoglobin and phosphate, adding approximately 18 mOsm. to body fluids. If the fraction of infused acid neutralized by exchange of hydrogen ions for sodium and potassium ions had been neutralized entirely by bicarbonate outside the radiosulfate volume, the resulting osmolarity expected would be 289 mOsm. per liter.

11.4 L. × 303.4 mOsm./L. = 3,455 mOsm.

(3,455 mOsm. + 18 mOsm.) ÷ (11.4 L. + 0.615 L.) = 289 mOsm.

If this fraction (51 per cent) had been neutralized by intracellular phosphate and proteinate buffers, with intracellular sodium and potassium ions exchanging for infused hydrogen ions, extracellular fluid would gain 184 mOsm. (0.51 × 360 mOsm.). Intracellular fluid would lose 92 mOsm. as weak acids of phosphate and proteinate replaced dissociated sodium and potassium salts of these anions. The net effect would be to increase osmolarity to:

(3,455 mOsm. + 18 mOsm. + 92 mOsm.) ÷ (11.4 L. + 0.615 L.) = 297 mOsm./L. and extracellular fluid volume to 0.72 liter.

4.47 L. × 303.4 mOsm./L. = 1357 mOsm. (in extracellular fluid initially)

1,357 mOsm. + 184 mOsm. = 1,541 mOsm. (after infusion)

1,541 mOsm. ÷ 297 mOsm./L. = 5.19 L.

5.19 L. − 4.47 L. = 0.72 L.

The osmolarity of plasma water (297.5 mOsm. per L., Table III) and the increment in radiosulfate volume (0.715 L., Table II) following acid infusion are in close agreement with the calculated

values. These calculations do not consider some contribution to neutralization of acid by intracellular bicarbonate expected in these cationic transfers, a contribution which we are unable to estimate.

If sodium and potassium entering extracellular fluid to neutralize 51 per cent of infused acid had come from apatite crystals of bone by the reaction suggested by Bergstrom (17),

$$—Ca—O—CO_3—Na + H^+ \text{ Anion}^- \rightarrow —Ca—\text{Anion} + Na^+ HCO_3^-$$

The net effect would be replacement of Na^+ and Cl^- for H^+ and Cl^- in extracellular fluid and an increase in extracellular solute of 184 mOsm. without a concomitant change in osmotically active solute within cells. Osmolarity of plasma water expected after infusion of acid would be:

(3,455 mOsm. + 18 mOsm. + 184 mOsm.) ÷ (11.4 L. + 0.615 L.) = 304 mOsm./L.

The expected increase in extracellular fluid volume would be

1,541 mOsm. ÷ 304 mOsm./L. = 5.07 L.

5.07 L. − 4.47 L. = 0.60 L.

That infused chloride is more than accounted for in erythrocytes and in the radiosulfate volume does not militate against this possibility since anion other than chloride might be substituted on the apatite crystal for sodium and carbonate (18).

These calculations ignore changes in water content of erythrocytes, changes in water and ion content of cerebrospinal fluid and perhaps gastrointestinal fluid, blood sampling and insensible water losses (19), factors which probably will not introduce important errors.

Combinations of the three possible mechanisms considered or of other mechanisms not considered could account for observed results. However, observed changes in extracellular fluid volume and plasma osmolarity agree closely with changes calculated by assuming that half of the infused acid is neutralized by exchange of extracellular hydrogen ions for intracellular sodium and potassium ions. This suggests that cells are the source of monovalent base entering extracellular fluid in *acute* acidosis rather than apatite crystals in bone as observed in more chronic, less severe, metabolic acidosis in rats (2).

Transfers of water, sodium, potassium, and hydrogen between cells and extracellular fluid of these magnitudes would reduce intracellular sodium concentration by a degree consistent with that observed in analysis of muscle in metabolic acidosis (20). Such transfers would reduce intracellular water, pH and bicarbonate concentration, leaving intracellular potassium concentration essentially unchanged. Hastings and Eichelberger (21) and Cotlove, Holliday, Schwartz, and Wallace (20) calculated a slight increase in muscle cell water in metabolic acidosis and a slight increase in potassium concentration in muscle cell water (20). Wallace and Hastings (22) calculated that intracellular pH of muscle did not change significantly in metabolic acidosis and that intracellular bicarbonate concentration decreased only slightly. Thus in some respects the cation and water transfers proposed are inconsistent with available information on changes in muscle water and electrolytes in acidosis produced by hydrochloric acid administration.

The cation and water transfers suggested as responsible for the increase in extracellular sodium, potassium and water observed in these experiments on nephrectomized dogs may also follow hydrochloric acid infusion in the presence of renal function. However, it would be expected that increments in extracellular water, sodium, potassium, and chloride would not be maintained in the presence of renal function. When renal function is maintained, it is well known that metabolic acidosis is usually associated with a contracted extracellular fluid volume.

C. Location of chloride not included in the radiosulfate volume and in erythrocytes

The increment in chloride in the radiosulfate volume and in erythrocytes accounts for slightly more than the chloride infused. Since the radiosulfate volume and erythrocytes contain only 80 to 85 per cent of the total body chloride (5), this would indicate that extracellular chloride concentration can increase 10 to 15 per cent without a corresponding increase in the amount of chloride located elsewhere than in the radiosulfate volume and in erythrocytes. While this suggests that 15 to 20 per cent of the total chloride may be either bound to extracellular solid structures or located intracellularly at unchanging concentration, it is probable that the methods are not sufficiently accurate to justify the conclusion that the magnitude

of this smaller part of the total chloride did not change.

SUMMARY

Severe metabolic acidosis has been produced in nephrectomized dogs by infusing 9 to 10 mM per Kg. of hydrochloric acid over a period of two hours. Changes in total extracellular sodium, potassium, bicarbonate, and chloride have been calculated from plasma concentrations and changes in the radiosulfate volume of distribution. These changes indicate that approximately 40 per cent of the infused acid is neutralized by extracellular bicarbonate, approximately 10 per cent by erythrocyte buffers, and approximately 50 per cent by diffusion of sodium and potassium into extracellular fluid. Exchange of extracellular hydrogen ions for intracellular sodium and potassium ions is suggested to account for the increment in extracellular sodium and potassium.

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COMPARISON OF WEDGED HEPATIC VEIN PRESSURE WITH PORTAL VEIN PRESSURE IN HUMAN SUBJECTS WITH CIRRHOSIS [1, 2]

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A method of assessing portal venous pressure in animals by wedging a cardiac catheter into a peripheral hepatic venule was described by Myers and Taylor (1) and by Friedman and Weiner (2). The application of this technique in humans has been reported by Myers and Taylor (1), Paton, Reynolds, and Sherlock (3), and Krook (4). It seems clear from the evidence presented that the presence of portal hypertension can be reliably predicted from the finding of an elevated wedged hepatic vein pressure (WHVP). Paton, Reynolds, and Sherlock have shown that in patients without liver disease WHVP measures only a few mm. Hg more than right atrial pressure (3). Likewise in non-cirrhotic liver disorders not associated with portal hypertension WHVP is normal (3). By contrast in a large number of cirrhotic subjects reported by the aforementioned three groups of investigators WHVP was invariably elevated to some degree.

This technique would be more valuable if it were known how closely the pressure measured in the wedged position corresponded to actual portal venous pressure (PVP). In cats (1) and in dogs (2) WHVP was found to be almost identical with PVP both in the normal animal and when both pressures had been elevated by epinephrine injection. In two human subjects with portal hypertension, PVP, measured postoperatively through a polyethylene tube leading from an omental vein, compared favorably with WHVP measured preoperatively (3). To our knowledge no other studies have been reported comparing WHVP and PVP in man.

This communication describes a number of such comparisons the preliminary results of which have been published in abstract form (5).

METHODS AND MATERIAL

We have followed the technique originally described by Warren and Brannon (6) and elaborated by Bradley, Ingelfinger, Bradley, and Curry (7) for catheterization of the hepatic vein. A cardiac catheter is passed from the median antecubital vein of either arm under fluoroscopic guidance into the right atrium. We have had the best results with moderately stiff catheters, No. 8 or No. 9 gauge, prepared straight except for the usual curved tip. Right atrial pressure is recorded with a Statham strain gauge located 5 cm. posterior to the sternal angle with the patient supine. The catheter is then turned so that the curved tip is pointed to the right and posteriorly in order to enter the inferior vena cava. This may require considerable manipulation in subjects with cirrhosis because of the unusual location of the caval orifice incident to hepatic enlargement. The catheter tip will usually enter a right sided hepatic vein if it is advanced 1 to 2 cm. beyond the cavo-auricular junction; if it does not do so the tip can be rotated anteriorly and the wall of the vena cava explored again. The catheter seldom enters the hepatic vein draining the left lobe. The right renal vein can be mistaken for an hepatic vein, however, it forms almost a right angle with the vena cava, it opens more caudad than most hepatic veins, in the lateral view it courses more posteriorly, and the blood withdrawn from it contains more oxygen. After entering an hepatic vein the catheter is advanced as far as possible into the liver substance until it is wedged in a small hepatic venule. When properly wedged it will usually be far out in the periphery of the liver (an anterior course can be checked fluoroscopically). When it is withdrawn slight tension will be required to free the tip from the wedged position from which it will spring suddenly. Unfortunately there is no way of being absolutely certain that the catheter is properly wedged; if it becomes lodged at a venous bifurcation and blood flow is not completely obstructed an accurate wedge pressure will not be recorded. For this reason it is advisable to make pressure records from

[1] Preliminary results reported before the Western Surgical Association, December 3, 1953, and the Western Section of the American Federation for Clinical Research, January 28, 1954.

[2] Aided by grants from the National Heart Institute, U. S. Public Health Service (H-1718) and the Los Angeles County Heart Association.

more than one site in the liver if possible. After the wedged position is attained pressure is measured and a continuous recording made as the catheter is withdrawn to the free hepatic vein. Inferior vena caval pressure and the gradient between inferior cava and the right atrium are recorded at the end of the procedure.

Since changes in the general level of venous pressure would be expected to produce equivalent changes in wedged hepatic vein and portal vein pressures regardless of the presence or absence of hepatic disease, WHVP and PVP have been expressed as net pressure above right atrial level. This has the effect of eliminating elevations in wedged pressure (and presumably portal pressure) such as have been shown to accompany right atrial pressure rises in heart failure (3). In this study when actual right atrial pressure could be recorded it was subtracted from measured wedged pressure and portal pressure to obtain WHVP and PVP. When actual right atrial pressure could not be measured (as in the patients at surgery) it was assumed to be zero and the strain gauge was located externally at the estimated right atrial level.

PVP was recorded by inserting a 21 gauge needle into the exposed portal vein perpendicularly to the long axis of the vein. The needle was connected to the strain gauge by a polyethylene tube of inside diameter 1.2 mm.

The 19 patients on whom the pressure comparisons were made had varying degrees of portal hypertension due to alcoholic cirrhosis. None had clinically evident ascites. Seventeen of them were operated on for the performance of a portacaval shunt though it was not performed in all instances. Two were operated on for other intra-abdominal procedures. The pressure comparisons were made in three different ways.

Group A. Two patients were operated upon for conditions other than their portal hypertension. At the time of surgery polyethylene tubing was secured in a mesenteric vein, brought to the outside through a stab wound in the anterior abdominal wall and kept patent by a slow infusion. This procedure has previously been performed without harm to the patient (3, 8). Three days postoperatively the hepatic vein was catheterized and WHVP was recorded, followed immediately by a pressure recording from the mesenteric vein *via* the polyethylene tubing. The strain gauge was maintained in the same position (5 cm. below the sternal angle with the patient supine) for all measurements. Both mesenteric vein pressure and wedged pressure were corrected by subtracting right atrial pressure.

Group B. Seven patients who underwent portacaval anastomosis were catheterized immediately preoperatively. WHVP was measured and corrected by subtracting right atrial pressure. From one to three hours later, in the operating room, the pressure in the portal vein itself was recorded. The estimated right atrial position (5 cm. posterior to the sternal angle) was used as a zero reference point for recording PVP.

Group C. Ten patients were catheterized immediately preceding operation for portacaval anastomosis. The catheter was left in the wedged hepatic vein and kept patent with a continuous slow infusion while the patient was taken to surgery. When the portal vein was exposed WHVP was recorded, followed immediately by PVP. Both recordings were made with the strain gauge at the same zero reference point—the externally estimated position of the right atrium. In five of the patients WHVP was also measured at the time of catheterization, prior to the administration of anaesthesia.

Complications

Catheterization of the hepatic vein appears to be an innocuous procedure. The danger points of cardiac catheterization (the coronary sinus, tricuspid valve, right ventricle) are avoided. Arrhythmias are minor or absent. There have been no untoward results in the authors' experience except for an occasional mild thrombophlebitis or infection at the cut-down site. The hepatic vein is easily entered in the normal subject. However, in patients with cirrhosis of the liver some difficulty may be encountered, necessitating an experienced operator and considerable manipulation of the catheter. We require an average of two minutes of fluoroscopic time per patient and have failed to enter the hepatic vein in 5 of over 70 attempts in cirrhotic patients.

RESULTS

In every instance WHVP was elevated and as the catheter was withdrawn to the free hepatic vein the abrupt drop in pressure characteristic of intrahepatic portal hypertension was seen (Figure 1). This abrupt pressure drop was never seen in patients who did not have hepatic cirrhosis.

Table I and Figure 2 depict the results of the pressure comparisons in the 19 patients. In every case WHVP was lower than PVP. However, in no instance was there a major discrepancy between the two readings.

In the two patients in Group A WHVP was 4 and 8 per cent below mesenteric vein pressure.

In the seven cases in Group B WHVP aver-

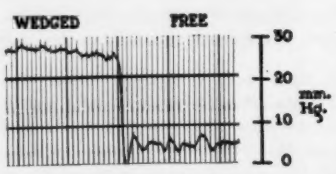

FIG. 1. ABRUPT DROP IN PRESSURE AS THE CATHETER IS WITHDRAWN FROM A "WEDGED" POSITION IN THE HEPATIC VEIN TO A "FREE" POSITION

TABLE I

Wedged hepatic, portal and mesenteric vein pressures in 19 subjects with cirrhosis

GROUP	CASE NO.	W.H.V.P. PRE-OPERATIVE (mm.Hg. above # rt. atrium)	W.H.V.P. AT SURGERY (mm.Hg. above ∫ rt. atrium)	P.V.P. AT SURGERY (mm.Hg. above ∫ rt. atrium)	MESENTERIC V.P. (mm.Hg. above # rt. atrium)
A	1	19.0*			21.0
	2	20.0*			21.0
B	3	12.0		14.0	
	4	17.0		20.0	
	5	20.0		21.0	
	6	21.0		24.0	
	7	26.0		29.0	
	8	28.0		31.0	
	9	28.0		32.0	
	Means	21.7		24.4	
C	10		13.0	14.0	
	11		13.0	15.0	
	12	15.5	13.5	17.0	
	13	19.5	15.0	20.0	
	14	20.5	17.0	22.0	
	15	24.0	21.0	26.0	
	16		21.0	29.0	
	17		22.0	29.0	
	18		23.0	25.0	
	19	27.0	24.0	30.0	
	Means		18.3	22.7	

* performed simultaneously with mesenteric vein pressure measurements - see text.

actual rt. atrial pressure measured and subtracted.

∫ strain gauge located at externally estimated rt. atrial level.

aged 11 per cent (range 5 to 15 per cent) below PVP.

In the 10 patients in Group C WHVP averaged 20 per cent (range 9 to 28 per cent) below PVP. A typical example is pictured in Figure 3. The greatest discrepancy observed was in a patient in whom WHVP measured 21 mm. Hg and PVP measured 29 mm. Hg.

In the combined group of 19 patients WHVP averaged 15.3 per cent below PVP.

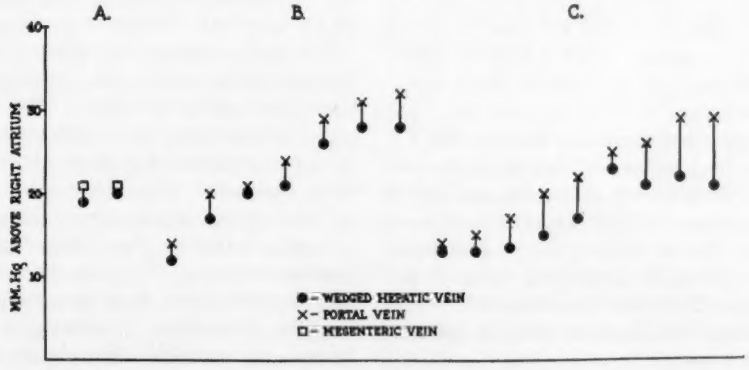

FIG. 2. COMPARISONS OF WEDGED HEPATIC VEIN AND PORTAL OR MESENTERIC VEIN PRESSURES IN THREE GROUPS OF PATIENTS WITH CIRRHOSIS

For description of the groups see text.

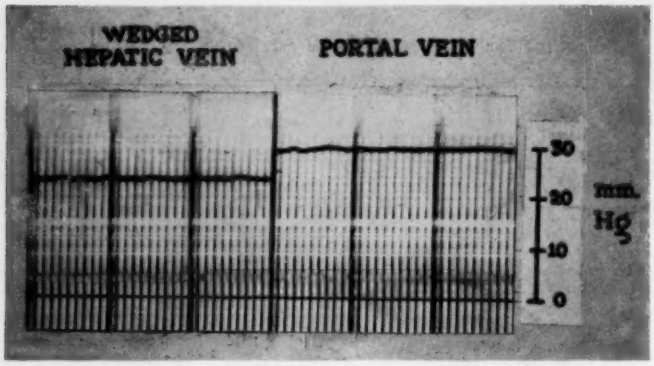

FIG. 3. COMPARISON OF WEDGED HEPATIC VEIN AND PORTAL VEIN PRESSURES MADE AT SURGERY

DISCUSSION

There are some theoretical objections to the pressure comparisons in Group A and B. In the two patients in Group A, the polyethylene was fixed in small mesenteric veins. It is possible that some degree of blood clotting occurred in this relatively static system in spite of the continuous infusion. A small clot near the end of the tubing might have interfered with accurate pressure recording.

In Group B the two pressure recordings were made under different circumstances. At the time of the PVP recording the patients were anaesthetized. Anaesthesia has been shown to lead to a fall in splanchnic blood flow in patients without liver disease (9, 10). This could be due either to splanchnic or intrahepatic vasoconstriction or both. If the former exceeded the latter, a fall in PVP would be expected. Such a situation might obtain in cirrhosis with a relatively fixed intrahepatic vasculature. PVP would then drop, accounting for the closer agreement between WHVP and PVP in our patients in Group B than in Group C. In fact, in each of the five patients in Group C in whom WHVP was measured both preoperatively and at surgery the second determination was lower, the mean drop being 15 per cent (Table I). This would be compatible with a fall in splanchnic blood flow secondary to splanchnic vasoconstriction.

Exact comparison between WHVP and PVP in the patients in Group B is also rendered unreliable by the possibility of small errors in positioning of the external zero reference point used for measuring PVP.

There seem to be no major objections to the validity of the comparisons in the patients in Group C. The same zero reference point was used for both pressures which were recorded by the same mechanism only seconds apart. It is true that a lateral pressure was recorded in the portal vein which neglects the kinetic energy of blood flow. The magnitude of this kinetic energy, though not measured, is assumed to be small because of the relatively low velocity of blood flow in the portal vein in cirrhosis.

Though there was considerable variability in the extent to which WHVP approached PVP, this variability was within a relatively narrow range and no instance of a marked discrepancy between the two pressures was encountered.

It is to be expected that pressure measured in the obstructed hepatic vein would be somewhat lower than portal pressure. The recording of portal pressure from the wedged catheter depends upon the presence of a static blood column between the portal inflow system and the catheter tip. Over this static column pressure can be transmitted without the usual decrement from vascular resistance. Although the wedged catheter does obstruct blood flow in a small segment of liver this obstruction is probably never complete because of collateral channels at or near the sinusoidal level. Hence WHVP should be consistently less the PVP. Hepatic arterial inflow into this relatively static vascular column could

raise catheter tip pressure to arterial levels were it not for the possibility of free backflow into the portal system when the latter's pressure is exceeded.

The fact that WHVP closely approaches PVP indicates that the majority of the abnormal resistance to blood flow in the cirrhotic liver lies between the wedged catheter tip and the nearest freely anastomosing vascular channels distal to it. If there are freely anastomosing channels in the sinusoidal area, it would follow that the major resistance is post-sinusoidal in the small hepatic venules. If, however, there is a diffuse reduction in anastomosing vessels throughout the liver lobule then no such inference can be drawn.

Even with the catheter free in the hepatic vein, there may be some obstruction to blood flow through that radicle. This may result in the recording of a higher pressure from the free hepatic vein than from the inferior vena cava. For this reason we have chosen not to express WHVP as the difference between the pressure levels in the wedged and free positions. The use of an external zero reference point for recording WHVP favors inconsistency between observers because of the difficulty in locating the point exactly at atrial level. Furthermore it provides no means of distinguishing between elevations in WHVP secondary to a generalized rise in venous pressure (*i.e.*, right heart failure) and elevations due to liver disease. The use of measured right atrial pressure as a zero reference point solves these problems. However, abnormal pressure gradients between inferior vena cava and right atrium will then be reflected in WHVP. We have found significant cavo-auricular pressure gradients in several patients with ascites (none in this series). In measuring PVP or WHVP we are chiefly interested in the resistance to blood flow through the liver, *i.e.*, the pressure gradient between portal system and inferior vena cava. Although it is true that resistance to blood flow in the inferior vena cava itself carries no implication as far as liver disease is concerned, it is equally true that any increment in portal pressure provided by this resistance is important in the development of esophageal varices. Since varices are connections between the portal and superior vena caval systems any factors contributing to a pressure differential between the two should be important. We have utilized right atrial pressure as a baseline for this reason. However, we believe inferior vena caval pressure should also be recorded since wedged pressure minus caval pressure gives the best picture of hepatic vascular resistance. It seems hardly necessary to emphasize that whether PVP or WHVP is used as an index of hepatic resistance the measurement is more valuable if it is accompanied by an estimate of liver blood flow.

A deterrent to the utilization of WHVP in a quantitative fashion is the lack of a definitive means of establishing that the catheter tip is properly wedged. We have occasionally, by continued manipulation, been able to advance the catheter tip further into the liver beyond the point at which it was originally thought to be wedged with the result that a higher WHVP was obtained. For this reason we emphasize the value of recording WHVP from more than one site.

CONCLUSION

From the data presented it appears likely that, in cirrhosis, WHVP represents a large and roughly predictable fraction of PVP. Further comparisons of these two measurements should establish whether this is true in all patients with cirrhosis.

Knowledge of the approximate portal pressure as determined by this technique has proven to be of clinical value in the diagnosis of obscure cases of cirrhosis (11), in assessing the need for portacaval anastomosis, in evaluating the merits of other procedures designed to ameliorate portal hypertension and in the diagnosis of extrahepatic portal hypertension.

SUMMARY

1. Portal vein pressure (PVP) and the pressure measured after wedging a catheter into a peripheral hepatic vein (WHVP) have been compared in 19 cirrhotic patients undergoing abdominal surgery.

2. WHVP correlated reasonably well with PVP in all instances. In 10 simultaneous comparisons WHVP averaged 20 per cent (range 9 to 28 per cent) below PVP.

3. In five patients WHVP averaged 15 per cent (range 13 to 23 per cent) lower at surgery than preoperatively.

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THE TRANSFER IN HUMANS OF DELAYED SKIN SENSITIVITY TO STREPTOCOCCAL M SUBSTANCE AND TO TUBERCULIN WITH DISRUPTED LEUCOCYTES [1, 2]

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It is possible to transfer regularly immunologically specific, generalized skin sensitivity of the delayed type to tuberculin in animals (1, 2) and in human subjects (3, 4) by means of an injection of leucocytes obtained from sensitive donors.

The cellular transfer system has been applied to the study of the streptococcal hypersensitive state in human subjects (5, 6) and the results parallel those obtained with tuberculin as the test material.

In the human subject the induced tuberculin skin sensitivity following leucocytic transfer is, in many respects, similar to that which occurs spontaneously following infection with the tubercle bacillus—namely, it is immunologically specific; it involves the entire skin of the recipient wherever tested; it is frequently as marked in intensity and occasionally maximal skin sensitization of the recipient has been accompanied by a systemic response.

The tuberculin sensitive state following cellular transfer differs from that occurring spontaneously, in that sensitization of some recipients by the former method may be evanescent and disappear entirely in a matter of days or weeks. However, many recipients following cellular transfer may remain sensitized for six to eight months and a few for as long as one year.[3]

A similar parallelism between the spontaneous skin sensitivity to streptococcal materials and that induced following cellular transfer cannot be drawn because there is no clear demonstration in man that the acquisition of positive skin reactions to streptococcal materials with age is directly referable to a specific hemolytic streptococcal infection (7).

The known factors which appear to condition the degree and the duration of the sensitive state following transfer, are the degree of sensitivity of the leucocyte donor and the amount of the leucocytes used. Moreover, leucocytes obtained from non-sensitive donors have not induced sensitization of the recipient.

In each of the studies on delayed bacterial sensitivity cited above, the leucocytes used have been intact and viable. In animals treatment of the leucocytes by freezing and thawing has consistently abolished the capacity to transfer delayed sensitivity to tuberculin (1, 2) and the treatment of the leucocytes by freezing and thawing (2, 8) or distilled water lysis (8) has also abolished the capacity to transfer serum antibody. Recently, successful transfer of contact sensitivity of the delayed type to 2, 4-dinitrochlorobenzene has been reported in animals using leucocytes disrupted by sonic vibration; however, attempts to extend this observation to other delayed sensitivities, including tuberculin sensitivity, were stated to be without success (9).

It is the purpose of this report to present data which indicate in the human, that successful transfer of delayed skin sensitivity to tuberculin and to partially purified preparations of the streptococcal M substance can be achieved regularly with suspensions of leucocyte components liberated, either by repeated freezing and thawing or by distilled water lysis. In addition the treatment of such disrupted leucocyte suspensions with the enzymes desoxyribonuclease (DNase)[4] or ribonuclease (RNase),[4] respectively, did not diminish the ca-

[1] Conducted under a contract recommended by the Commission on Streptococcal Diseases, Armed Forces Epidemiological Board, Office of the Surgeon General, U. S. Army.

[2] Presented in part at the Forty-Sixth Annual Meeting of the American Society for Clinical Investigation, Atlantic City, New Jersey, May 4, 1954.

[3] Observations made on recipients subsequent to publication of References 3 and 5.

[4] The abbreviations DNase for desoxyribonuclease, DNA for desoxyribonucleic acid, RNase for ribonuclease, and RNA for ribonucleic acid are used in this report.

pacity to induce sensitization of the recipient to tuberculin.

MATERIALS AND METHODS

Isolation of leucocytes

Detailed descriptions of the technic of leucocyte isolation have been reported (3, 5). Briefly, the leucocytes are isolated from the heparinized venous blood of appropriately sensitive donors following the acceleration of the erythrocyte sedimentation rate with bovine fibrinogen, Fraction I (Armour). The supernatant plasma suspension of leucocytes is collected and centrifuged,[5] the plasma pipetted off and the isolated leucocytes pooled.

Repeated washing of the pooled leucocytes, to elute contaminating erythrocytes and traces of plasma, was not carried out in this study since it was shown in earlier studies that neither erythrocytes, nor plasma, nor serum, but only leucocytes, and only those obtained from markedly sensitive donors, were capable of inducing delayed sensitivity (3, 5). Attention is paid to the yield of leucocytes, since it has been observed that if the challenging skin test material is injected at a site remote from the site of leucocyte injection, then at least 0.1 cc. of leucocytes are required for the successful transfer of generalized delayed sensitivity (3, 5, 6). For the experiments to be described 120 to 180 cc. of blood is employed with an approximate yield of 0.3 to 0.6 cc. leucocytes for each transfer.

Treatment of leucocytes

The pooled leucocytes are treated by one of the following procedures, and the progress of leucocyte disruption is followed to its completion by parallel giemsa and Feulgen stained (10) smears.

1. Distilled water lysis. For complete lysis of the cell population in a pool of 0.3 to 0.6 cc. leucocytes, at least 1 to 2 cc. of distilled water for each 0.04 cc. leucocytes is necessary. For 0.4 cc. leucocytes this would amount to a 10 to 12 cc. solution, which is then placed in a 37° C. water bath for two to four hours with frequent agitation by pipetting to insure adequate contact and to break up the floccules of nucleoprotein.

Microscopic observations made during the course of leucocytolysis indicate that this procedure results initially in the rupture of the cell membrane with leakage of the cytoplasmic components into the supernatant portion of the solution. Subsequently the nuclear membrane ruptures and the nuclear material unravels into skeins of a viscous sediment. The latter takes on a purple color when stained by the Feulgen method and is Feulgen-positive, indicating the presence of highly polymerized Desoxyribonucleic acid (DNA) for which this stain is specific (11). The cytoplasmic material in the supernatant is colored green and is Feulgen-negative. In the skeins of nucleoprotein sediment there are caught what appear to be Feulgen-negative cytoplasmic ghosts from which the nucleus has been extruded en masse or from which the nuclear material has been leached in the lytic process. There is usually no Feulgen-positive material in the cytoplasmic ghosts; however, in a few, flecks of Feulgen-positive material may remain. The number of what appear to be intact leucocytes or intact round cell nuclei will vary from none, to rare, to one per 50 to 85 high power fields. The nucleoprotein sediment is centrifuged at 1,000 RPM for 15 minutes and resuspended for injection in 2 cc. of the cytoplasmic supernatant. In the experiments detailed in Table I, it is this nucleoprotein sediment resuspended in only 2 cc. of cytoplasmic solution which is injected into the negative recipient. In the experiments detailed in Table II, it is the remainder of the cytoplasmic supernatant solution which is injected into the negative recipient. It contains occasional fragments of Feulgen-positive nuclear material but not any intact cells or cytoplasmic ghosts.

2. Freezing and thawing. The pooled leucocytes are resuspended in 3 cc. normal saline and frozen in a dry-ice-90 per cent alcohol solution and are kept frozen solid for two hours before the first thawing in a 37° C. water bath. The leucocytes are then alternately rapidly frozen for 10 to 15 minutes and thawed in the 37° C. water bath for 10 to 15 minutes, for a total of 7 to 10 cycles. The end point of each freezing cycle is taken as the time of appearance of a central nipple in the solid mass rather than any rigid time unit. The total number of freezing and thawing cycles for any particular experiment is governed by the microscopic evidence of the completeness of the disruption of the leucocytes. Microscopic observations made during the course of freezing and thawing are similar in most respects to those detailed under distilled water lysis. There are, however, fewer cytoplasmic ghosts noted following this method of disruption.

3. Freezing and thawing plus enzymes. The leucocytes are frozen and thawed as in Section 2 above and then treated for one hour in a 37° C. water bath with the enzymes desoxyribonuclease (DNase) or ribonuclease (RNase), respectively. Periodic pipetting of the mixture is employed to insure adequate contact between enzyme and substrate.

a) Crystalline pancreatic desoxyribonuclease (DNase) (Worthington 1 × Crystallized, Lot D-424) (12) is dissolved in 10 cc. normal saline containing 0.2 cc. normal human serum albumin (Cutter) to a concentration of 1 mgm. per cc. equivalent to 120,000 units by viscosimetric assay. The method of viscosimetric assay used is that of Johnson, Goger, and Tillett (13). The leucocytes to be treated with DNase are frozen and thawed in saline to which has been added 100 mgm. $MgSO_4$. The Mg^{++} is added to inhibit the citrate ion (bovine fibrinogen contains approximately 40 to 50 per cent citrate) and to activate the DNase (14). One mgm. of DNase (120,000 units) is added to the frozen and thawed leucocytes and

[5] The gentle centrifugation, important for the isolation of intact, viable cells, was replaced in this study with centrifugation for 5 minutes at 1,000 RPM and 10 minutes at 2,600 RPM.

allowed to act for one hour at 37° C. water bath before injection of the suspension into the recipient. Paralleling the gross change in viscosity following the addition of DNase, the skeins of DNA fibrillae are no longer discernible microscopically and their Feulgen-positive quality is lost. Structures resembling intact leucocytes or round cell nuclei—and were interpreted as such after lysis or freezing and thawing—disappear as do the cytoplasmic ghosts following dissolution of the protective matrix of DNA fibrillae. This would indicate that such structures were neither intact nor viable leucocytes since DNase does not attack living cells (15). It is rare to find an intact, viable leucocyte in DNase treated suspensions.

b) Crystalline ribonuclease (Worthington, Salt and Protease-free) (Lot R-522-4) (16, 17) is dissolved in 0.0005 N HCl at a concentration of 2 mgm. per cc. at a pH of 2 and to 0.5 cc. of this solution is added 0.5 cc. Sorenson's buffer solution. The final concentration of ribonuclease is 1 mgm. per cc. at a pH of 7.

Citrate (1 cc. of 2.5 per cent solution sodium citrate for each 10 cc. blood) is substituted for heparin as the anticoagulant for leucocyte isolation in the ribonuclease experiments since heparin inhibits ribonuclease activity (18). In addition, since the various lots of RNase were found to have 0.1, 0.2, and 0.03 units of DNase activity per milligram of RNase by viscosimetric assay, the pooled leucocytes are suspended in equal volumes (1.5 cc.) of citrate and normal saline, adequate to inhibit the DNase activity (14) before freezing and thawing.

When RNase is added to frozen and thawed leucocyte suspensions no grossly visible change in viscosity occurs. The extracellular DNA remains highly polymerized and Feulgen-positive and the cytoplasmic elements retain the green color on Feulgen stain.

Test materials

Streptococcal M substance.[6] Partially purified preparations of M substance prepared from Type I hemolytic streptococcus are used. The method of preparation and the properties of M substance (Lot A15) have been described by Lancefield and Perlmann (19). Lyophilized M substance is dissolved in saline so that each skin test dose in 0.1 cc. solution contains 0.01 mgm. (equivalent to 1.4 γ nitrogen) or 0.002 mgm. (equivalent to 0.028 γ nitrogen). The use of the term "M substance" in this report is descriptive and such usage is not meant to imply chemical or immunological purity.

Old tuberculin (O. T.) (Bureau of Laboratories, Department of Health, N. Y. C.) Second test strength—1:1000 dilution equivalent to 0.1 mgm. tuberculin for each skin test dose of 0.1 cc.

Purified protein derivative (PPD) (Parke, Davis & Company.) Second test strength— equivalent to 0.005 mgm. PPD for each skin test dose of 0.1 cc.

Criteria for reading skin tests

The criteria for reading delayed cutaneous reactions to old tuberculin, purified protein derivative or streptococcal M substance are the same as those used in earlier studies (3, 5) and reactions are designated from 0 to 4+, with each (+) equivalent to approximately 10 × 10 mm. edema and erythema.

Selection of donors and recipients

1. Streptococcal M substance as the test material. The group is comprised of 10 M-positive leucocyte donors with marked delayed skin sensitivity to streptococcal M substance at 24 hours and 48 hours after skin testing and 14 M-negative recipients with no cutaneous reaction to the same concentration of M substance on at least two occasions just prior to transfer. The concentration of M substance used for screening the donors and recipients for nine transfers was 0.01 mgm. (1.4 γ nitrogen) and for four transfers was 0.002 mgm. (0.028 γ nitrogen) per skin test dose, respectively. Recipient RiD (Table II) was tested on only one occasion just before transfer. One additional M-negative subject was used as a donor for control studies.

The 10 donors and 14 recipients of the leucocytes were patients hospitalized on the wards of the Third (NYU) Medical Division of Bellevue Hospital and ranged in age from 40 to 60 years with four exceptions. The subjects had no clinical or historical evidence of recent hemolytic streptococcal infection or its delayed complications and were not cachectic nor gravely ill.

2. Tuberculin as the test material. The group is comprised of three tuberculin positive donors of leucocytes with marked delayed skin sensitivity to the tuberculin patch test and 21 tuberculin negative recipients with no skin sensitivity to either second strength old tuberculin 0.1 cc. (0.1 mgm.) or second strength PPD 0.1 cc. (0.005 mgm.) on at least two occasions just prior to transfer. The 16 tuberculin negative recipients making up the test and control groups of the enzyme treated leucocyte suspension studies, were negative to both old tuberculin and PPD on two occasions just prior to transfer.

The donors and recipients of the leucocytes in this group were all healthy male medical students who ranged in age from 22 to 26 years. There was one female student nurse in the group. Each of the medical students had been followed by the author for at least two years and many for three years prior to transfer. In each there had been no clinical or roentgenographic evidence of tuberculosis and each negative recipient had remained tuberculin negative when tested on repeated occasions during this period.

The student nurse (MaS) originally tuberculin negative (PPD 0.005 mgm.) in March, 1951, was given BCG in April, 1951 and became positive to tuberculin (+++ PPD 0.005 mgm.) in June, 1951. When subsequently retested in May, 1952, June, 1953 and October, 1953, prior to transfer, skin reactions to tuberculin (PPD 0.005 mgm. and O. T. 1.0 mgm.) were negative on each occasion.

Method of transfer

When the treatment of the leucocytes has been brought to completion (lysis, freeze-thawing, freeze-thawing plus

[6] Kindly supplied by Dr. R. C. Lancefield.

enzyme) it is that respective suspension which is injected intradermally and/or subcutaneously into the skin of the shoulder of the respective negative recipient and such depots are not disturbed thereafter. The following day (18 to 24 hours after injection) the skin of the volar surface of the opposite forearm is challenged with the same concentration of the specific testing material to which the recipient had not been sensitive and the leucocyte donor sensitive. Cutaneous reactions to the test material are recorded at 6 hours, 24 hours and 48 hours. The evolution and subsequent course of the induced sensitivity is followed by repeating the skin test at approximately weekly intervals thereafter, until reversion to the original negative state occurs.

In the three transfers done with the cytoplasmic supernatant solution of distilled water lysed leucocytes, the much larger volume is injected intramuscularly in the deltoid or buttock, and the forearm challenged as above.

RESULTS

Studies with mechanically disrupted leucocytes

A. Streptococcal M substance as the test material. Partially purified preparations of Streptococcal M substance (Type I) were chosen as a streptococcal test material because of the close resemblance of the behaviour of M substance in the induced M-sensitive state following cellular transfer in humans to the behaviour of tuberculin in tuberculin sensitivity. In particular, intradermal injection of M substance into reactors with cutaneous hypersensitivity produces predominantly delayed skin reactions of the tuberculin type; moreover, repeated skin testing with M substance does not by itself cause the appearance of delayed reactions of the tuberculin type (5, 6).

It is to be emphasized that the preparations of M substance used in these studies are only partially purified, and that delayed skin reactions to such preparations may be due to some contaminating material not yet identified. It is possible that the reactions induced in the leucocyte recipients may also be due to trace amounts of a contaminating material, rather than to the M antigen itself (see ViF, Table III).

There has been no attempt here to define the contribution of contaminating materials in the M substance, or of its type specificity, to the delayed reactions observed. While precise, quantitative immunochemical technics can be utilized in the study of antigens having related skin-sensitizing serum antibody systems (20–25), the cellular transfer system is not susceptible to the adaptation of such technics, at this time, since the substance(s) in the leucocyte responsible for the transfer of delayed sensitivity has yet to be identi fied and quantitated.

Therefore, the delayed skin reaction to partially purified preparations of streptococcal M substance is here used as an indicator, and it is the underlying mechanism of the cellular transfer system which is being evaluated.

The results of the transfer of delayed sensitivity

TABLE I

TRANSFER OF DELAYED M-SENSITIVITY WITH LYSED LEUCOCYTES USING THE NUCLEOPROTEIN SEDIMENT IN CYTOPLASMIC SOLUTION.

M-Negative Recipient		M-Positive Donor	Donor's Degree Sensitivity	Approx. Volume WBC For Lysis ± cc.	Maximum Cutaneous M-Sensitivity In Recipient			Duration of Sensitized State in Recipient Days
					Days After Transfer	24 Hrs. After Skin Test	48 Hrs. After Skin Test	
NiR	Exp. 1	PeB	3+	0.5	-	0	0	-
	Exp. 2	FiT	4+	0.3	14	2+	2+	24
	Exp. 3	TaS	4+	0.5	1	2+	2+	23
Hel	Exp. 4	PeB	3+	0.5	-	0	0	-
	Exp. 5	FiT	4+	0.08	-	0	0	-
	Exp. 6	FiT	4+	0.4	9	3+	1+	>21
InG	Exp. 7	JoC	4+	0.5	2	4+	4+	> 7
AnC	Exp. 8	InG	4+	0.5	2	2+	1+	5
JoB	Exp. 9	WiW	4+	0.3	2	3+	1+	17

to streptococcal M substance in negative recipients of the nucleoprotein sediment in a cytoplasmic solution of lysed leucocytes appear in Table I.

In six consecutive instances the M-negative recipients of the nucleoprotein and cytoplasmic components of lysed leucocytes obtained from M-positive donors, developed varying degrees of delayed skin sensitivity to M substance.

Negative recipient NiR failed to develop sensitivity (Exp. 1) following the injection of lysed leucocytes obtained from a moderately sensitive donor (PeB) yet subsequently developed sensitivity (Exp. 2) following the injection of lysed leucocytes, albeit in a lower dosage, obtained from a markedly sensitive donor (FiT). The induced sensitivity in recipient NiR had disappeared when retested for the third time, 24 days following the successful transfer of Experiment 2. Sensitivity to M substance in recipient NiR was induced again (Exp. 3) following the injection of lysed leucocytes obtained from another markedly sensitive donor (TaS) and disappeared again on the retest of the 23rd day after transfer.

A similar failure to develop sensitivity was observed in negative recipient HeI (Exp. 4) when lysed leucocytes from the same moderately sensitive donor (PeB) were used. In addition recipient HeI (Exp. 5) also failed to develop sensitivity when the lysate of an insufficient volume (0.08 cc.) of leucocytes obtained from the markedly sensitive donor (FiT, Exp. 2 above) was deliberately chosen to be given. However, this negative recipient (HeI) finally developed M-sensitivity (Exp. 6) following the injection of the lysate from an adequate volume of leucocytes obtained from the same markedly sensitive donor (FiT).

These results confirm the equal importance of a minimum dosage of leucocytes (at least 0.1 cc. and preferably 0.2 to 0.3 cc. using this method) and a markedly sensitive donor in the successful transfer of delayed sensitivity. Similar results have been observed in earlier studies in the human using intact, viable leucocytes (3, 5).

Negative recipient InG (Exp. 7) developed marked sensitivity to M substance within two days after the injection of lysed leucocytes obtained from markedly sensitive donor (JoC). At the height of this maximum response, on the third day after transfer, leucocytes were in turn obtained from this formerly negative recipient (InG), lysed and injected into a second negative recipient AnC (Exp. 8). Two days after this serial transfer AnC developed minimal sensitivity (2+) to M substance which disappeared entirely when tested again at the end of five days.

In an effort to exclude the contribution of the few intact leucocytes observed, along with the possibility of other intact leucocytes hidden in the tangled skeins of nucleoprotein sediment, cytoplasmic supernatant solutions of leucocyte lysates were prepared as described and assessed for the capacity to transfer delayed sensitivity to M substance. The results appear in Table II.

In three consecutive instances the M-negative

TABLE II

TRANSFER OF DELAYED M-SENSITIVITY WITH LYSED LEUCOCYTES USING CYTOPLASMIC SUPERNATANT SOLUTION.

M-Negative Recipient	M-Positive Donor	Volumes		Maximum Cutaneous M-Sensitivity in Recipient			Duration of Sensitized State in Recipient Days
		WBC Lysed ± cc.	Supernatant Injected cc.	Days After Transfer	24 Hrs. After Skin Test	48 Hrs. After Skin Test	
RiD	FiT*	0.3	10	15	3+	±	> 60
ThW	JoC*	0.5	8	1	2+	2+	15
AyC	WiW	0.6	7	9	3+	1+	> 20

* The cytoplasmic supernatants of leucocytes from donors FiT and JoC were prepared at the same time and from the same leucocytes that were lysed for use in Exp. 2 and 7 of Table I.

TABLE III

TRANSFER OF DELAYED M-SENSITIVITY WITH LEUCOCYTES DISRUPTED BY FREEZING AND THAWING.

M-Substance Status of Donor	M-Negative Recipient	Approx. Volume WBC ± cc. Frozen and Thawed	Maximum Cutaneous M-Sensitivity in Recipient			Duration of Sensitized State in Recipient Months
			Days After Transfer	24 hrs. After Skin Test	48 hrs. After Skin Test	
Positive (AdL)	WiJ	0.4	6	3+	2+	>10
Positive (JoS)	WiL	0.3	14	3+	2+	> 1.5
Positive (CaR)	EdM†	0.3	4	4+	4+	8
Positive (JoB)	EdM	0.6	3	1+	1+	Unknown
Negative (BeH) Exp. 1	ViF	0.7	26	4+	3+	> 5
Negative (BeH) Exp. 2	FrK	0.2	-	0	0	-
Negative (BeH) Exp. 3	BeH	0.2	-	0	0	-

† Developed effusion ankle joint coincident with maximal skin sensitivity.

recipients of the cytoplasmic supernatant prepared from the lysed leucocytes of M-positive donors, developed delayed skin sensitivity to M substance.

The results of the transfer of delayed sensitivity to streptococcal M substance in negative recipients of frozen and thawed leucocytes appear in Table III.

In four consecutive instances the M-negative recipients of repeatedly frozen and thawed leucocytes obtained from M-positive donors, developed varying degrees of generalized delayed cutaneous sensitivity to M substance.

One negative recipient (EdM) developed a tender, swollen ankle joint coincident with the development of the induced positive skin reaction to M substance in the forearm one day after transfer. The swelling, warmth and tenderness of the joint increased and on the third day after transfer, 10 cc. of fluid was aspirated. The fluid was clear, yellowish, and viscous with rare round cells on smear. During this period the patient was afebrile with a normal leucocyte count, an ESR = 7 mm. (Wintrobe), ASL titre = 75 units per cc., C-reactive protein negative and serial electrocardiograms were normal. The involvement of the joint receded rapidly after aspiration. Whether this event was related to the induced skin sensitivity to M substance or was a coincidence is not known.

In control studies the attempt to transfer M-sensitivity with frozen and thawed leucocytes obtained from an M-negative donor (BeH, Exp. 1) resulted in the development of marked M sensitivity in one negative recipient (ViF). Since previous attempts to transfer delayed sensitivity with intact viable leucocytes obtained from negative donors were unsuccessful (3, 5) this experiment was repeated. Leucocytes were obtained from the same negative donor (BeH, Exp. 2) frozen and thawed and injected into another negative recipient (FrK) who did not subsequently develop M-sensitivity. In addition leucocytes were again obtained from the same negative donor (BeH, Exp. 3) frozen and thawed and injected back into herself (BeH) without the subsequent development of M-sensitivity.

The reason for the exceptional response of recipient ViF is not known. It may possibly be related to the fact that he received an amount of leucocytes (0.7 cc.) three and one-half times greater than recipients FrK (0.2 cc.) and BeH (0.2 cc.) and the induced sensitivity observed may be in this instance due to trace amounts of contaminating materials rather than to the M antigen itself, as indicated above.

Thus in thirteen consecutive instances the M-negative recipients of components of leucocytes obtained from M-positive donors and dis-

TABLE IV

TRANSFER OF DELAYED TUBERCULIN SENSITIVITY WITH LEUCOCYTES DISRUPTED BY FREEZING AND THAWING.

Tuberculin Status of Donor	Tuberculin Negative Recipient	Approx. Volume WBC ± cc. Frozen and Thawed	Maximum Cutaneous Tuberculin Sensitivity in Recipient			Duration of Sensitized State in Recipient Months
			Days After Transfer	24 Hrs. After Skin Test	48 Hrs. After Skin Test	
Positive (HuC)	MaS	0.25	22	4+	3+	> 9
Positive (MaS)	ArC	0.50	49	2+	1+	> 7
Positive (EdC)	JoS	0.25	20	2+	1+	3
Negative (FrG)	GeE	0.4	-	0	0	-
Negative (GeE)	FrG	0.5	-	0	0	-

rupted by means of two different technics developed generalized delayed cutaneous sensitivity to partially purified preparations of M substance.

In three consecutive attempts to transfer M sensitivity with disrupted leucocytes obtained from the same M-negative donor one was successful and two were not successful.

B. Tuberculin as the test material. It was felt desirable to substantiate the observations detailed above with the tuberculin system, since it is the prototype of delayed bacterial hypersensitivity and is a useful control in evaluating the results of transfer studies of the streptococcal hypersensitive state. Therefore, the studies just described using partially purified preparations of the streptococcal M substance as the test material were repeated using tuberculin as the test material. The isolated leucocytes were disrupted by repeated freezing and thawing as described above.

The results of the transfer of tuberculin sensitivity with the components of repeatedly frozen and thawed leucocytes appear in Table IV.

In three consecutive instances the tuberculin negative recipients of repeatedly frozen and thawed leucocytes obtained from tuberculin positive donors, developed varying degrees of generalized delayed cutaneous hypersensitivity to tuberculin.

The first tuberculin negative recipient (MaS) developed moderate (2+) tuberculin sensitivity when tested six days after an injection of frozen and thawed leucocytes obtained from tuberculin positive donor (HuC) which reached its maximum (4+) when tested on the 22nd day after transfer. At the height of this maximum response on the 22nd day after transfer, leucocytes were in turn obtained from this formerly negative recipient (MaS), frozen and thawed and injected into a second negative recipient (ArC). Thirty days after this serial transfer ArC developed minimum sensitivity to tuberculin (+) which reached its maximum (++) at the end of 49 days. At present, eight months after transfer ArC's sensitivity is minimal and has just about disappeared.

In two consecutive instances attempts to transfer tuberculin sensitivity to negative recipients (GeE and FrG, Table IV) with frozen and thawed leucocytes obtained from negative donors were unsuccessful.

Thus the observation that components of disrupted leucocytes are capable of transferring delayed cutaneous sensitivity to streptococcal M substance is substantiated when tuberculin is used as the test material.

Enzyme treatment of frozen and thawed leucocytes

The reproducible nature of the results just described presented the opportunity to introduce additional variables into the donor system with the reassurance afforded by a relatively stable test system.

For this purpose the isolated leucocytes are frozen and thawed in the manner described and to the suspension of leucocyte components a specific enzyme, desoxyribonuclease (DNase) or ribonuclease (RNase), is added.

TABLE V

TRANSFER OF DELAYED TUBERCULIN SENSITIVITY WITH FROZEN AND THAWED AND ENZYME TREATED LEUCOCYTES.

Tuberculin Positive Donor	Tuberculin Negative Recipient	Approx. Volume WBC ± cc Frozen and Thawed	Treatment of Disrupted Leucocytes	Maximum Cutaneous Tuberculin Sensitivity in Recipient			Duration Sensitized State in Recipient Months
				Days After Transfer	24 Hrs. After Skin Test	48 Hrs. After Skin Test	
HuC	FrG	0.4	-	24	1+	±	>7
	PaC	0.4	DNase	56	3+	1+	>7
	RiH	0.5	-	61	3+	2+	>7
	KlB	0.5	DNase	56	4+	3+	>7
AmS	IrF	0.6	-	61	4+	4+	>7
	RuF	0.6	DNase	7	3+	3+	2
	MaT	0.7	-	55	2+	3+	>7
	StS	0.7	DNase	28	2+	2+	>7
HuC	FrF	0.6	-	21	3+	3+	4
	BeG	0.8	RNase	35	2+	1+	>4
	MaP	0.6	-	11	1+	1+	>4
	MaR	0.6	RNase	22	3+	2+	>4
AmS	HoK	0.5	-	11	3+	1+	>3
	HaB	0.5	RNase	74	3+	2+	>3
	StC	0.5	-	53	2+	1+	>3
	HoL	0.5	RNase	20	3+	2+	>3

The results of the action of DNase upon the extracellular DNA can be interpreted with some confidence, since the Feulgen stain is highly specific and permits concrete visual evidence of depolymerization of all available DNA (11). There is not such a reliable histochemical technique for RNA and its depolymerization, hence there is some uncertainty about the extent and degree of depolymerization of the RNA in these experiments. This reservation exists despite the advantage taken of all precautions known to be propitious for the action of RNase (18, 26). In addition, a residue of RNA is known to remain undigested even when a large excess of RNase is employed (26).

For this group of experiments sixteen additional tuberculin negative recipients were selected and had prepared for them on different occasions, suspensions of the constituents of repeatedly frozen and thawed leucocytes obtained from one of two tuberculin positive donors. Each of eight alternate recipients received an injection of such untreated suspensions; of the remainder, four recipients received DNase treated suspensions and four recipients received RNase treated suspensions. The results are summarized in Table V.

In sixteen consecutive instances each negative recipient developed generalized delayed cutaneous tuberculin hypersensitivity and the pattern of response appeared similar in the untreated and enzyme treated groups. Of the control group of eight recipients, three became moderately and five markedly sensitive to tuberculin. Of the DNase treated group of four, one became moderately and three markedly sensitive to tuberculin, and of the RNase treated group of four, one became moderately and three markedly sensitive to tuberculin.

Comparison of sensitivity induced with disrupted leucocytes to that induced with intact leucocytes

In each instance described above the induced delayed sensitivity to M substance or to tuberculin in negative recipients following the injection of disrupted leucocytes is similar in most respects to that observed in human subjects following the injection of intact, viable leucocytes (3, 5) and to that seen in the spontaneously sensitive individual (6).

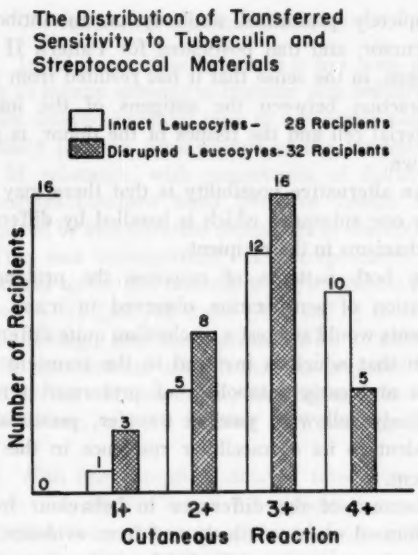

FIGURE 1.

One aspect of this similarity is graphically represented in Figure 1 which plots the distribution in terms of degree of sensitivity achieved in 60 consecutive recipients of the transfer of delayed sensitivity in humans, where the results in 28 recipients [7] using intact, viable leucocytes (3, 5) are compared with the results in the 32 recipients of this report using disrupted cells.

DISCUSSION

With the finding that it is regularly possible in the human to transfer bacterial sensitivity of the delayed tuberculin type by means of the components of disrupted leucocytes, it became feasible to attempt identification of the particular leucocyte component(s) concerned in the tissue sensitization process and gain some clue to the mechanism whereby sensitization is achieved. A step in this direction is the application of enzymatic techniques to selectively eliminate one cellular component from the whole at a time.

The sequence of events following cellular transfer with intact or disrupted leucocytes, suggests that this system may have analogies to other biological systems where self-replication of a foreign material is fostered by the cells of the host. Examples of the latter are to be found in the behaviour of the DNA of the pneumococcal transforming principle (*27*, 28) and in the intracellular replication of viral particles in microbial (29), plant (30) and animal cells (31–34). This similarity is suggested by the observation that relative to the considerable cell mass of the adult human recipient, an infinitesimal amount of donor cell material is capable of altering the reaction capacity of the entire skin surface of the recipient for periods in many instances as long as six to eight months and in a few as long as one year. This response has heretofore been achieved only following prolonged, intimate contact with considerable numbers of the specific bacterium (tubercle bacillus, streptococcus) and the tissues of the host (6).

Moreover, unlike the passive transfer of preformed serum antibody the transfer of leucocytes, intact or disrupted, appears to require the active participation of the recipient before maximum and sustained effects may be achieved. Although the response is variable for each recipient, a review of the data collected in this and earlier studies (3, 5), comprising a total of 60 successful individual transfers, indicates that two broad, but distinct, temporal patterns of recipient response exist following the transfer of delayed sensitivity. The patterns are schematically represented in Figure 2.

It will be recalled that all recipients are skin-tested within a day after cellular transfer and thereafter at approximately weekly intervals. In this diagram the magnitude (0 to 4+) of the reaction to each individual skin test is plotted

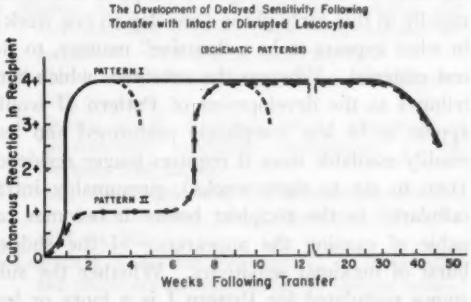

FIGURE 2.

[7] Unpublished results in two recipients of the transfer of M-sensitivity (each graded 4+) using lysed leucocytes which were incompletely disrupted are included among the "intact" leucocyte group.

against the time of application of that skin test following cellular transfer.

The recipients with a response which approximates that shown in Pattern I exhibit maximum sensitivity (4 +) to the first or second skin test, applied within a day or a week following transfer, The recipients with a response which approximates that shown in Pattern II exhibit sensitivity early, as in Pattern I, but the reactions to each of the skin tests are minimal (1 +), for intervals which vary from two to six to eight weeks following transfer. At the end of this variable lag period there is an abrupt appearance of maximum sensitivity (4 +) in response to the respective weekly skin test. In both patterns, maximum sensitivity may be evanescent and disappear entirely, or it may gradually wane in intensity when the recipient is subsequently retested, or it may persist for prolonged periods on subsequent retesting, as indicated in Figure 2. Since this sequence of events does not occur in relation to the application of one skin test, but represents a progressive change in the magnitude of the response of the recipient to successive skin tests, it differs from the phenomenon described by Andrewes, Derick, and Swift (35), where a delayed flare-up of an intradermal reaction occurs in the same skin test site with the passage of time.

The observation of two distinct temporal patterns following the transfer of an immunological response suggests that two different substances or different phases of the same substance, each related to a separate pattern of response, may be present in the leucocytes of the donor.

The substance which contributes to the development of Pattern I would appear to be more completely preformed and readily available in amounts sufficient to cause the negative recipient to respond rapidly in this early phase (one day to one week), in what appears to be a "passive" manner, to the test material. Whereas the substance which contributes to the development of Pattern II would appear to be less completely preformed and less readily available since it requires longer residence (two to six to eight weeks), presumably intracellularly, in the recipient before it becomes capable of causing the appearance of the sudden burst of maximal sensitivity. Whether the substance postulated for Pattern I is a more or less completely preformed antibody or an antibody precursor, and that postulated for Pattern II an antigen, in the sense that it has resulted from the interaction between the antigens of the intact bacterial cell and the tissues of the donor, is not known.

An alternative possibility is that there may be only one substance which is handled by different mechanisms in the recipient.

In both patterns of response the prolonged duration of sensitization observed in many recipients would suggest a mechanism quite different from that which is involved in the transient effects and early catabolism of preformed serum antibody following passive transfer, presumably incident to its extracellular residence in the recipient.

Because of the difference in behaviour from preformed serum antibody and from evidence, as yet only suggestive, afforded by two attempts at serial transfer, it is reasonable to suppose that this substance(s) may be taken up by the cells of the recipient which are concerned with the production of delayed sensitivity and in turn alter their metabolism for variable periods, perhaps in a manner similar to that by which the specific bacterium, some time in the past, altered the metabolism of the cells of the donor in this regard.

Hence, our attempts to identify the components of the leucocyte responsible for this effect have focussed on the cell components of the donor with self-replicating potentialities. Assuming the substance responsible for the transfer of delayed sensitivity was the DNA of the nucleus, then treatment with the enzyme DNase might be expected to abolish this capacity and similarly if assumed to be the RNA, then treatment with RNase might be expected to abolish it. These assumptions were tested as reported and the results were negative.

From such negative results, conclusions are limited and tentative; however, it would appear that the mechanism responsible for the transfer of delayed sensitivity does not depend upon the highly polymerized DNA or highly polymerized RNA components of the donor cell. The working hypothesis detailed above, however, retains its usefulness in aiding the design of further experimental tests of its validity.

SUMMARY AND CONCLUSIONS

1. In 32 consecutive instances it has been possible in human subjects to transfer skin sensitivity of the delayed bacterial type to tuberculin and to partially purified preparations of the streptococcal M substance, with components of disrupted leucocytes obtained from sensitive donors and prepared by two different techniques of disruption.

This was accomplished with streptococcal M substance as the test material using leucocytes prepared by: a) Distilled water lysis, and b) freezing and thawing; and accomplished with tuberculin as the test material using leucocytes prepared by: a) Freezing and thawing, b) freezing and thawing plus DNase, and c) freezing and thawing plus RNase.

2. With one exception, delayed sensitivity did not develop in five negative recipients when the disrupted leucocytes were obtained from donors without sensitivity to the test material.

3. The induced delayed sensitivity to tuberculin and to streptococcal M substance in negative recipients following the injection of components of disrupted leucocytes is similar, in most respects, to that which has been observed in humans following the injection of intact viable leucocytes.

4. Treatment of disrupted leucocyte suspensions with the enzymes DNase or RNase did not diminish the capacity to transfer delayed sensitivity.

5. A working hypothesis concerning the mechanism whereby the transfer of delayed sensitivity may be mediated, is discussed.

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THE EXTRARENAL RESPONSE TO ACUTE ACID-BASE DISTURBANCES OF RESPIRATORY ORIGIN [1]

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The plasma electrolyte changes in acute respiratory acidosis and alkalosis cannot be explained solely on the basis of renal compensatory mechanisms. In acute respiratory acidosis, renal conservation of base is accomplished by means of increased excretion of ammonia and titratable acid (1) as well as by enhanced renal reabsorption of bicarbonate bound base (2, 3). However, under acute conditions, the degree of base saving effected by these means cannot account for the increment of bicarbonate bound base observed. This initial increment of bicarbonate bound base in the extracellular fluid which these renal tubular mechanisms then maintain, must be supplied from extrarenal sources. Similarly, extrarenal mediation must pertain in acute respiratory alkalosis, since altered renal excretion of base cannot alone account for the extracellular electrolyte changes which occur (4). Furthermore, renal mechanisms cannot explain completely the changes in plasma concentration of chloride, phosphate, and organic acids observed in acute respiratory acid-base disturbances.

The nature of the extrarenal compensation for acid-base imbalances has been studied by various approaches. In metabolic acidosis, direct tissue studies reveal alteration of the content of tissue phosphates (5), of sodium and potassium of bone and soft tissues (5), and of carbon dioxide stores of bone and muscle (6). The observed changes are interpreted to be evidence of direct tissue compensation. In addition, the buffering of infused acid or alkali cannot be accounted for by blood buffers alone (7–9), nor by the buffer capacity of the extracellular fluid (10). Further, altered organic acid metabolism has been invoked as capable of modifying significantly the response of the organism to an alkali invasion (11).

Fewer studies exist as to the nature of the tissue response to an acid-base disturbance of respiratory origin. In this regard three lines of evidence can be cited. First, studies in respiratory acidosis indicate that of the total carbon dioxide gained, 80 to 90 per cent is accounted for by extravascular sites, largely bone and muscle (12, 13); conversely, a loss of comparable degree from tissues occurs in respiratory alkalosis. Second, direct muscle analyses indicate changes in cell base in respiratory acid-base imbalance (14, 15), which may be taken to reflect tissue compensation. Finally, that tissue electrolytes may be made available for extracellular buffering, has been postulated. Thus, various investigations have revealed changes in carbon dioxide capacity (16, 17) of blood, and alterations in plasma concentration of various electrolytes of such magnitude and direction to be compatible with transfer of these constituents across an extracellular boundary (18, 19).

The present study is an attempt to delineate more precisely and completely the nature and extent of the tissue contribution to the extracellular compartment in the compensation for an acute respiratory acidosis or alkalosis.

Nephrectomized dogs were used to eliminate any factor of renal compensation. By the measurement of extracellular fluid volume and of plasma and red blood cell electrolyte concentration, an estimate could be obtained of transfer of ions into or out of the readily available extracellular fluid volume.

Tissue contribution to compensation in the extracellular fluid for respiratory acidosis or alkalosis was found to be mediated mainly by three means: Transfer of chloride across the erythrocyte membrane; shifts of base across an extracellular boundary other than the red blood cell

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membrane; and significant alterations in organic acid content of the extracellular fluid.

EXPERIMENTAL PROCEDURE

A total of 29 experiments was performed on 24 male dogs, ranging in weight from 14.6 to 35.9 Kg. Anesthesia was induced and maintained by sodium pentobarbital, given intravenously. Bilateral nephrectomy was performed by flank approaches. Blood was obtained from a needle indwelling in a femoral artery. A polyethylene catheter was inserted into the femoral vein, and used for administration of the various materials infused. An endotracheal airway with inflatable rubber cuff was introduced.

Following these procedures, appropriate amounts of sucrose, radiosulfate, and/or radiochloride (Cl^{36}) were infused in a manner previously reported from this laboratory (20). The total time for infusion including washout with 50 to 100 ml. of 3 per cent glucose in water never exceeded 15 minutes. In a total of three experiments the sucrose blank of plasma was followed over several hours of respiratory acidosis or alkalosis, and increase in blank with time was found to be comparable with that reported by Swan, Madisso, and Pitts (20). Appropriate blank correction was applied.

In some experiments radiosodium (Na^{22}) was infused. In such cases this material was given intravenously, 12 to 18 hours prior to the experiment to assure adequate equilibration. The dose used was usually 50 μc, equivalent to about 8×10^6 cpm. Sufficient carrier was added to make a final concentration of 0.107 M NaCl. Correction was applied for radiosodium lost into the urine prior to nephrectomy.

An equilibration time of two and one-half hours from the midpoint of infusion was used for sucrose, radiosulfate, and for radiochloride. In 16 experiments, sucrose and radiosulfate were used simultaneously to estimate readily available extracellular fluid volume.

Subsequent to equilibration time, blood was collected from the indwelling arterial needle at hourly intervals for two to three hours. The amounts drawn each time varied between 25 and 40 ml.

In acidosis experiments, following the control periods the dog breathed gas mixtures, usually containing 20 per cent CO_2 and 80 per cent O_2 for two to six hours. In a few experiments the gas mixture contained 12 per cent CO_2 and 88 per cent O_2. The gas was administered by attaching a Douglas bag containing the mixture to a non-rebreathing Digby-Leigh valve which was connected to the endotracheal tube.

In alkalosis experiments, hyperventilation was maintained for two to four hours by means of a Palmer-type respiration pump attached to the endotracheal tube. In these experiments room air was used. In a few studies the initially induced period of acidosis or alkalosis was followed by another set of control periods, during which the dog was allowed to breath room air spontaneously; these then were succeeded by two further periods during which the condition opposite to that of the initial experimental periods was induced: hyperventilation after initial CO_2 breathing or CO_2 breathing after hyperventilation.

ANALYTICAL METHODS

Plasma was analyzed for Na, K, Cl, CO_2, and PO_4 by methods described previously (21). Values for pH were determined on samples of whole blood in the manner described by these authors. Lactate of plasma was estimated by the method of Barker and Summerson (22). Sucrose, radiosulfate, and Cl^{36} of plasma were determined by methods described in a previous report from this laboratory (20). The gamma-emission of Na^{22} of plasma was measured in a lead-shielded scintillation counter, containing a Thallium activated sodium iodide crystal, and a steel filter for elimination of weak beta-emission. Determinations were performed on a 2 ml. wet sample in metal planchettes. A suitable Na^{22} standard in saline (0.107 M) was used. Comparison with standards in plasma revealed no significant difference. Adequate sample and background counts were performed to assure a consistency of counting to within ± 2 per cent. By the analytical methods used, complete separation of Na^{22} and Cl^{36} could be achieved. No such complete separation could be accomplished in the case of radiosulfate and Na^{22}, since the precipitates of radiosulfate gave excessively high counts, due to contamination with Na^{22}. Therefore, radiosulfate was not used in the few experiments in which Na^{22} was infused. Plasma volume was estimated by the T-1824 dye-dilution technique (23). A measure of total proteins of plasma was obtained by the copper sulfate method of Van Slyke and his associates (24). Hematocrit was measured in Wintrobe tubes, spun at 4,000 RPM in a centrifuge of 17 inch diameter for 30 minutes. Whole blood analyses for Na and K were performed using an internal standard flame photometer. Whole blood CO_2 content was determined according to the method of Van Slyke and Neill (25). Whole blood chlorides were determined on a Van Slyke-Hawkins protein-free filtrate (26), an aliquot of which was analyzed by the method of Van Slyke and Hiller (27).

CALCULATIONS

Symbols and formulas used are given in detail in the Appendix. Total amounts of ions in the readily available extracellular fluid (ECF) were calculated from data of plasma concentration, plasma volume, and radiosulfate space ($VS^{35}O_4$), with appropriate factors applied for Gibbs-Donnan distribution and water content of plasma. Total amounts of ions in the total red blood cell mass were calculated from data of plasma concentration, whole blood concentrations, plasma volume, and hematocrit. The increments or decrements of total extracellular or red cell ions were calculated in each experiment by averaging the total of each ion species in control periods and in periods of respiratory acidosis or

alkalosis. Increments are recorded as $+\Delta$mM, decrements as $-\Delta$mM. In experiments in which both acidosis and alkalosis were induced in the same animal, each pair of periods (control, acidosis, or alkalosis), was compared to the immediately preceding pair.

RESULTS

Readily available ECF in respiratory acidosis and alkalosis in nephrectomized dogs

Figure 1 shows the plasma disappearance curves of sucrose and radiosulfate of several representative experiments. Similar curves were obtained in all animals. It should be noted that the plot is a semi-logarithmic one. The vertical line indicates the time at which acidosis or alkalosis was induced.

It is apparent that in all cases a straight line could be fitted through all points, since the logarithm of plasma concentration decreases linearly with time.[3] This linearity is compatible with constant clearance from unchanging volume of distribution (*29*). The conclusion, therefore, seems warranted that neither the sucrose space (VS) nor radiosulfate space ($VS^{35}O_4$) changed after induction of respiratory acidosis or alkalosis. In 16 experiments the volumes of distribution of sucrose and radiosulfate were measured simultaneously. The average ratio $VS/VS^{35}O_4$ was found to be 0.90. This value is in good agreement with the value (0.91) recently given by Swan, Madisso, and Pitts (*20*). On the basis of the findings of the same authors, the radiosulfate space was selected as a measure of extracellular fluid. In 8 experiments where the radiosulfate space was not measured, its equivalent was obtained by multiplying the sucrose space by 1/0.90. This procedure appears permissible, since this ratio has been shown to remain unchanged in respiratory acidosis or alkalosis. From the data presented the conclusion is drawn that extracellular fluid volume did not change in respiratory acidosis or alkalosis.

[3] In the nephrectomized animal the slopes of the curves of disappearance of radiosulfate and sucrose from plasma are dependent on extrarenal removal of these substances from the extracellular fluid. This extrarenal loss, such as slow metabolic breakdown, secretion into the gastrointestinal tract, *etc.*, prevents these disappearance curves from reaching a plateau. Recent experiments by Mulrow, Oestreich, and Swan (*28*) show that the slopes of disappearance from plasma of radiosulfate and sucrose remain unchanged in the nephrectomized animal for as long as 30 hours. The fact that no plateau is reached and that no "flattening out" occurs, strongly indicates that decrease in plasma concentration of these substances is not indicative of progressive increase in extracellular fluid volume but of constant extrarenal removal of these substances from an unchanging volume of distribution.

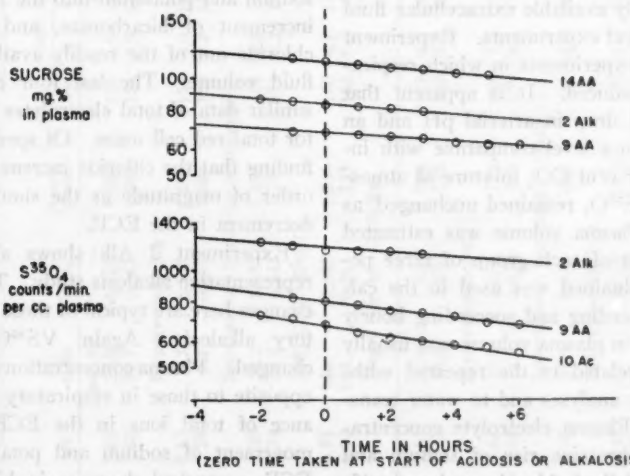

FIG. 1. DISAPPEARANCE OF RADIOSULFATE AND SUCROSE FROM PLASMA IN NEPHRECTOMIZED DOGS, SUBJECTED TO RESPIRATORY ACIDOSIS OR ALKALOSIS

TABLE I

Experiments illustrating the effect of acute respiratory acidosis and alkalosis on distribution of electrolytes

Elapsed time	pH	Art. pCO_2	$VS^{35}O_4$	Plasma volume	Conc. in plasma water				Quantity in readily available extracellular space				Quantity in total red blood cell volume			
					Na	K	Cl	HCO_3	Na	K	Cl	HCO_3	Na	K	Cl	HCO_3
min.		*mm. Hg*	*ml. H_2O*		*mM/L.*				*mM*				*mM*			
150	7.30	46.5	4,610	753	155	3.2	108	23.4	682	14	517	113	83	5	38	9
210	7.30	43.2	4,610	753	153	3.8	107	21.2	676	17	512	107	80	5	38	8
270	7.29	43.2	4,610	753	150	4.0	107	20.7	668	18	516	100	79	5	37	7
275		20% CO_2—80% O_2														
330	6.84	173	4,610	678	158	4.3	108	30.6	701	19	517	147	91	7	50	15
390	6.86	179	4,610	678	159	7.6	105	32.7	701	33	498	156	103	8	56	19
450	6.89	173	4,610	678	161	8.0	103	33.4	713	35	492	159	100	8	55	19
510	6.81	199	4,610	655	162	7.8	106	32.5	717	34	502	155	96	8	54	18
570	6.84	176	4,610	655	163	6.9	104	31.0	723	30	497	148	89	6	51	17
630	6.82	183	4,610	655	167	5.7	105	30.4	739	25	500	145	94	7	52	15
		Dog 10 Ac—20.5 Kg.						ΔmMols.	+41	+13	−14	+45	+15	+2	+15	+9
150	7.39	43.3	5,270	948	162	3.8	114	26.6	820	19	617	146	125	8	86	16
210	7.35	46.4	5,270	948	159	3.9	113	26.3	803	20	619	144	128	8	81	16
270	7.40	39.9	5,270	948	161	4.0	112	26.3	814	20	613	141	121	7	76	15
275		Hyperventilation														
330	7.74	6.6	5,270	854	159	3.3	113	8.9	800	17	619	49	116	8	74	7
390	7.75	6.6	5,270	854	156	3.3	117	8.6	787	17	644	47	100	8	61	3
450	7.73	6.6	5,270	854	159	2.8	120	8.1	803	14	659	44	94	8	57	3
		Dog 2 Alk—23.6 Kg.						ΔmMols.	−16	−4	+25	−97	−22	0	−17	−12

Changes in electrolyte distribution in respiratory acidosis and alkalosis

In Table I are presented data on electrolyte changes in the readily available extracellular fluid obtained in two typical experiments. Experiment 10 Ac is one of 18 experiments in which respiratory acidosis was induced. It is apparent that there was a marked drop in arterial pH and an elevation of pCO_2 to a level compatible with inspiration of a 20 per cent CO_2 mixture at atmospheric pressure. $VS^{35}O_4$ remained unchanged, as described above. Plasma volume was estimated once at the midpoint of each group of three periods. The value obtained was used in the calculations for the preceding and succeeding hourly period. A decrease in plasma volume was usually observed, possibly related to the repeated withdrawals of blood for analyses and to water transfer into red cells. Plasma electrolyte concentrations showed a progressive rise of sodium and potassium, a slight fall of chloride, and a significant increase of bicarbonate. The next four columns show total electrolytes in the extracellular fluid, and in the lowermost row are presented the increments and decrements of these ions in the ECF. It is evident that there was a transfer of sodium and potassium into the ECF, a significant increment of bicarbonate, and a movement of chloride out of the readily available extracellular fluid volume. The last four columns illustrate similar data of total electrolytes and net transfers for total red cell mass. Of special interest is the finding that the chloride increment is of the same order of magnitude as the simultaneous chloride decrement in the ECF.

Experiment 2 Alk shows similar data in a representative alkalosis study. The pH and pCO_2 changes here are typical of those in severe respiratory alkalosis. Again, $VS^{35}O_4$ remained unchanged. Plasma concentration data show changes opposite to those in respiratory acidosis; the balance of total ions in the ECF demonstrates a movement of sodium and potassium out of the ECF, a marked decrease in bicarbonate, and a significant increment of extracellular chloride. In this case, red cell data again reveal a chloride

TABLE II

Experiments illustrating the effect of acute respiratory alkalosis and acidosis, in the same animal, on distribution of electrolytes, including phosphate and lactate

Elapsed time	pH	Art. pCO_2	$Vs^{35}o_4$	Plasma volume	Conc. in plasma water						Quantity in readily available extracellular space						Quantity in total red blood cell volume			
					Na	K	Cl	HCO_3	PO_4	Lact.	Na	K	Cl	HCO_3	PO_4	Lact.	Na	K	Cl	HCO_3
min.		*mm. Hg*	*ml. H_2O*		*mM/L.*						*mM*						*mM*			
150	7.42	29.9	6,545	1,487	155	3.9	116	20.8	1.4	1.0	972	25	793	142	9	6				
210	7.45	26.7	6,545	1,487	154	3.9	115	19.8	1.5	1.2	969	26	781	136	10	8				
215		Hyperventilation																		
270	7.57	6.7	6,545	1,284	151	3.9	124	7.5	1.0	5.9	960	25	850	47	6	39				
330	7.54	6.1	6,545	1,284	150	3.5	125	6.9	0.7	6.7	952	22	854	43	5	44				
335		Spontaneous breathing of room air							ΔmMols.		−15	−2	+65	−94	− 4	+35				
390	7.30	33.2	6,545	1,281	154	3.8	115	17.2	1.6	3.1	974	24	785	118	10	20				
450	7.34	33.2	6,545	1,281	154	4.2	116	19.2	1.9	1.2	980	27	793	132	13	8				
455		20% CO_2—80% O_2							ΔmMols.		+21	+2	−63	+80	+ 6	−28				
510	6.87	146	6,545	1,233	160	4.7	113	28.0	3.4	1.4	1,006	29	777	190	22	9				
570	6.92	143	6,545	1,233	163	6.5	113	30.0	4.8	0.2	1,028	41	783	205	31	1				
		Dog 9 Alk-Ac—35.9 Kg.							ΔmMols.		+40	+9	− 9	+72	+15	− 9				
150	7.41	36.6	3,670*	718	158	4.7	110	23.8	2.1	1.6	555	16	423	91	8	6	102	8	59	14
210	7.37	36.6	3,670	718	158	4.5	112	22.0	2.0	1.9	555	16	428	84	8	7	118	9	62	13
215		Hyperventilation																		
270	7.61	10.0	3,670	706	154	4.2	114	11.5	0.7	8.5	546	15	438	44	3	31	108	8	54	6
330	7.63	10.0	3,670	706	150	4.7	113	11.6	0.8	6.4	535	13	434	44	3	24	100	9	49	5
335		Spontaneous breathing of room air							ΔmMols.		−14	−2	+10	−44	− 5	+21	−6	0	− 9	− 8
390	7.40	29.9	3,670	655	154	4.5	114	19.0	2.0	2.7	543	16	437	74	7	10	95	8	48	8
395		20% CO_2—80% O_2							ΔmMols.		+ 2	+2	+ 1	+30	+ 4	−18	−9	−1	− 4	+ 2
450	6.85	160	3,670	592	161	5.5	111	28.5	5.3	2.1	567	21	427	109	19	8	102	9	62	18
510	6.81	176	3,670	592	161	7.8	111	29.0	6.5	1.9	567	28	427	111	24	7	98	8	59	17
		Dog 14 Alk-Ac—21.0 Kg.							ΔmMols.		+24	+9	−10	+36	+15	− 2	+5	0	+13	+10

* $S^{35}O_4$ space estimated on the basis of sucrose space.

TABLE III

Net transfers of ions in acute respiratory acidosis

Experiment no.	Weight Kilo.	$Vs^{35}O_4$ ml. H_2O	$V_{sucrose}$ ml. H_2O	Balance in readily available extracellular space—mM Na^+	K^+	Cl^-	HCO_3^-	PO_4	Lact.
1 Ac	25.0	4,680*	4,220	+ 8	+ 2	−25	+34		
2 Ac	14.9	3,150*	2,840	+22	+ 5	−10	+25		
3 Ac	17.0	4,025*	3,625	+22	+ 1	0	+32		
4 Ac	17.2	3,805*	3,425	+32	+ 4	−10	+24		
5 Ac	19.5	3,645	3,640	+16	+10	− 2	+30		
6 Ac	22.0	4,025	3,345	+15	+ 5	0	+36		
7 Ac	19.9	3,855	3,455	+22	+10	−16	+42		
8 Ac	21.1	4,415	3,805	+47	+ 7	− 9	+66		
8 A	17.0	2,780*	2,505	+27	+ 5	− 8†	+25		
9 Ac	23.5	3,255	3,340	+20	+ 5	+ 5†	+13		
10 Ac	20.5	4,610	—	+41	+13	−14†	+45		
11 Ac	28.1	5,370	4,700	+53	+ 7	− 8†	+55		
12 Ac	19.6	4,305	4,320	+24	+15	+ 8†	+39		
13 Ac	25.4	5,670	5,440	+29	+26	−19†	+43		
9 Alk-Ac	35.9	6,545	6,210	+40	+ 9	− 9	+72	+15	−9
11 Ac-Alk	23.6	5,040	—	+15	+13	−22	+38	+ 5	−1
13 Alk-Ac	14.6	3,625	—	+ 9	+ 2	−14†	+29	+ 6	+2
14 Alk-Ac	21.0	3,670*	3,300	+24	+ 9	−10†	+36	+15	−2

* $S^{35}O_4$ space estimated from sucrose space.
† Red blood cell data obtained.

change of comparable magnitude but opposite direction to that in the ECF.

It is of importance that only about half of the decrement in extracellular bicarbonate can be accounted for by the observed concomitant changes in sodium, potassium, and chloride. A similar but smaller discrepancy was evident in respiratory acidosis. This discrepancy was considered too large to be explained by alterations in known cations; therefore, the anions phosphate and lactate were studied in subsequent experiments.

The experiments listed in Table II illustrate two additional significant features. In these experiments both alkalosis and acidosis were induced in the same animal, with appropriate control periods interposed. The data show a marked rise of lactate associated with a slight fall of phosphate in respiratory alkalosis, and a significant increase in phosphate with concomitant fall in lactate in periods of respiratory acidosis. These data reduce the anion deficits formerly observed. Furthermore, the total amounts of ions transferred into or out of the readily available extracellular fluid are of comparable magnitude to those observed in separate experiments of acidosis or alkalosis. The net transfers of ions are reversible and reestablishment of control conditions occurs to a reasonable extent after the pCO_2 has been restored to normal. This therefore illustrates the ready mobility of a fraction of body electrolytes in

TABLE IV

Net transfers of ions in acute respiratory alkalosis

Experiment no.	Weight Kilo.	$Vs^{35}O_4$ ml. H_2O	$V_{sucrose}$ ml. H_2O	Balance in readily available extracellular space—mM Na^+	K^+	Cl^-	HCO_3^-	PO_4	Lact.
1 Alk	22.3	4,040	3,555	−15	− 4	+17†	− 63		
2 Alk	23.6	5,270	4,390	−16	− 4	+25†	− 97		
3 Alk	20.9	4,755	4,150	−19	− 2	+12†	− 87		
5 Alk	24.1	6,400	5,965	−22	− 5	+20	−110		
6 Alk	22.5	4,575	3,940	−11	− 4	+13†	− 51		
7 Alk	19.9	4,470	3,695	−16	− 2	− 5†	− 95		
9 Alk-Ac	35.9	6,545	6,210	−15	− 2	+65	− 94	− 4	+35
11 Ac-Alk	23.6	5,040	—	− 3	−22	+43	− 79	−11	+32
12 Alk	16.4	2,685*	2,415	− 7	+ 9	+ 9†	− 60	+ 3	+27
13 Alk-Ac	14.6	3,625	—	−16	+ 1	+19†	− 58	− 6	+15
14 Alk-Ac	21.0	3,670*	3,300	−14	− 2	+10†	− 44	− 5	+21

* $S^{35}O_4$ space estimated from sucrose space.
† Red blood cell data obtained.

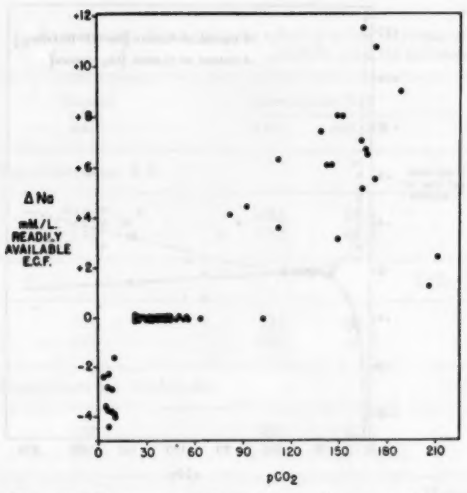

FIG. 2. TRANSFER OF SODIUM PER LITER OF READILY AVAILABLE EXTRACELLULAR FLUID, AS A FUNCTION OF ARTERIAL pCO_2

Each point represents a separate experiment. The values at zero on the ordinate scale represent control periods.

contributing to buffering within the extracellular fluid.

Experiment 14 Alk-Ac further demonstrates a similar lability of red cell electrolytes. Again, the extracellular chloride changes are of similar magnitude but opposite direction to those in the red cells.

In Tables III and IV are presented summaries of electrolyte transfers to or from the readily available extracellular fluid in all experiments performed. Shown are the results of 18 observations in respiratory acidosis and 11 observations in respiratory alkalosis. The consistency of the above described changes is evident. Variations in the absolute amounts of ions transferred in individual experiments are largely related to differences in the size of the extracellular fluid volumes and to differences in severity of the acidosis or alkalosis, *i.e.*, the extent of alteration of arterial pCO_2 (*vide infra*).

In a total of 8 acidosis experiments and 8 alkalosis experiments, red cell data were obtained. In the majority of observations the ΔCl of total red cell mass was of similar magnitude, but opposite sign to that of the ECF.[4]

[4] However, it should be pointed out that *in vivo* studies of transfers of ions across the red cell membrane have certain limitations. The possible sources of errors are: 1) Changes in total body hematocrit may not be closely reflected by the observed peripheral hematocrit (30); 2) errors derived from estimating red cell concentra-

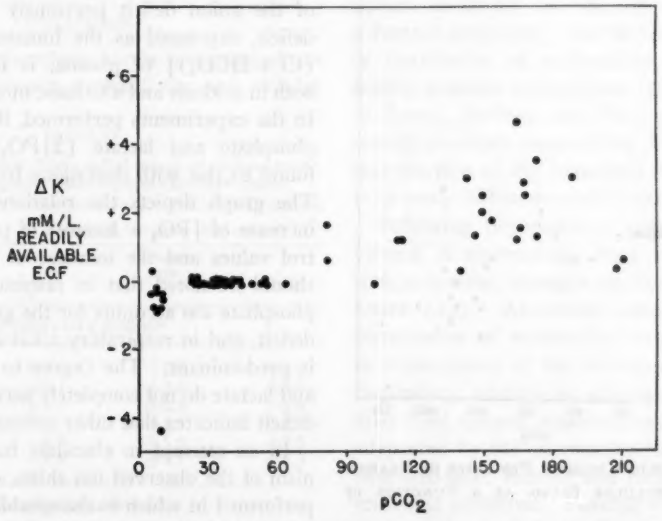

FIG. 3. TRANSFER OF POTASSIUM PER LITER OF READILY AVAILABLE EXTRACELLULAR FLUID, AS A FUNCTION OF ARTERIAL pCO_2

Each point represents a separate experiment. The values at zero on the ordinate scale represent control periods.

In Figure 2 the ΔNa per liter extracellular fluid is plotted as a function of arterial pCO_2. Each point represents a separate experiment. The points massed at zero on the ordinate scale represent pCO_2 values of control periods. It is evident that there is a direct and fairly linear relationship between the amounts of sodium transferred into or out of one liter of extracellular fluid, and deviations of pCO_2 from control values.

A relationship similar in direction but smaller in magnitude is presented for ΔK per liter extracellular fluid in Figure 3. Here again increasing amounts of base are transferred into the ECF with increasing arterial pCO_2.

Figure 4 depicts the interrelationship between ΔCl per liter ECF and arterial pCO_2. In this case the ΔCl per liter ECF is inversely related to deviations of pCO_2 from control values, increasingly more chloride disappearing from the extracellular fluid as the pCO_2 is raised.

tion of ions from whole blood, plasma, and hematocrit determinations may result in apparent transfers; 3) new cells which may be released into the circulation may not have the same average composition as those already in the blood stream (31).

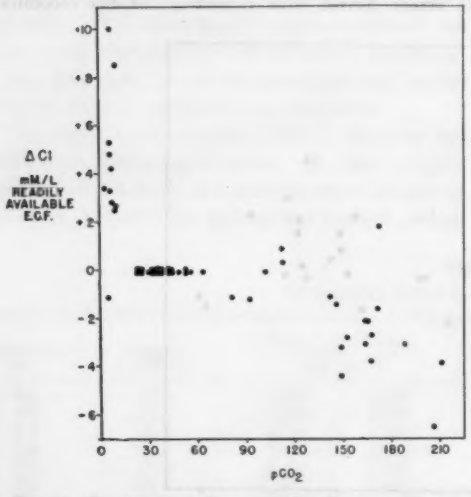

FIG. 4. TRANSFER OF CHLORIDE PER LITER OF READILY AVAILABLE EXTRACELLULAR FLUID, AS A FUNCTION OF ARTERIAL pCO_2

Each point represents a separate experiment. The values at zero on the ordinate scale represent control periods.

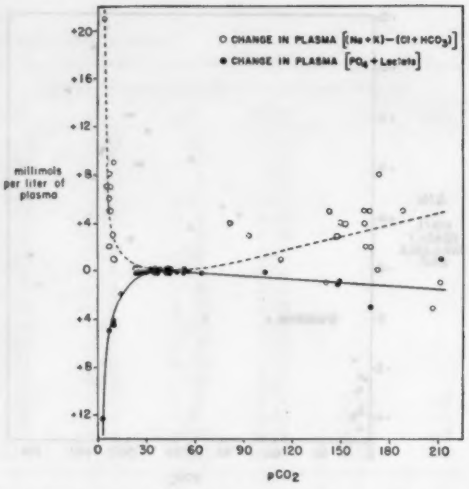

FIG. 5. VARIATIONS IN ANION DEFICIT, AS A FUNCTION OF ARTERIAL pCO_2

The points at zero on the ordinate scale represent control periods. The anion deficit $(Na + K) - (Cl + HCO_3)$, and the sum of plasma phosphate and lactate of control periods are arbitrarily taken as zero. Each point represents one experiment. The two curves are drawn free-hand.

Figure 5 illustrates the manner in which phosphate and lactate contribute to the diminution of the anion deficit previously discussed. This deficit, expressed as the function $[(Na + K) - (Cl + HCO_3)]$ of plasma, is found to increase both in acidosis and alkalosis, more so in the latter. In the experiments performed, the sum of plasma phosphate and lactate ($\Sigma[PO_4 + \text{lactate}]$) was found to rise with deviations from control pCO_2. The graph depicts the relationship between the increase of $[PO_4 + \text{lactate}]$ of plasma above control values and the increase of anion deficit. It should be noted that in respiratory acidosis the phosphate ion accounts for the greater part of this deficit, and in respiratory alkalosis the lactate ion is predominant. The degree to which phosphate and lactate do not completely account for the anion deficit indicates that other anions are involved.

In an attempt to elucidate further the mechanism of the observed ion shifts, experiments were performed in which exchangeable pools of sodium and chloride were determined. In some experiments the volume of distribution of radiochloride (VCl^{36}) was estimated. Table V illustrates ob-

TABLE V

Experiments illustrating the effect of acute respiratory acidosis or alkalosis on the exchangeable pool of sodium and chloride, and the volumes of distribution of radiochloride

Elapsed time min.	Exchangeable Na^+		Exchangeable Cl^-		$V_{Cl^{36}}$	
	mM	mM/Kg.	mM	mM/Kg.	ml. H_2O	% Body wt.
Experiment no. 8 A			Control			
150	684	40.4	508	30.3	4,170	24.7
210	713	42.2	500	29.6	4,100	24.3
			20% CO_2—80% O_2			
270	684	40.1	498	29.5	4,180	24.7
330	692	40.8	522	30.9	4,380	25.9
Experiment no. 14 Alk-Ac			Control			
150	994	47.3	586	27.9	5,090	24.2
210	1,002	47.8	603	28.7	5,170	24.6
			Hyperventilation			
270	997	47.4	603	28.7	5,075	24.2
330	969	46.1	606	28.9	5,155	25.5

servations in typical experiments. Table VI shows the deviations from control values in all acidosis and alkalosis experiments. The small and inconsistent changes observed are not regarded as significant.

DISCUSSION

Qualitatively, the observations presented above on ion shifts between extracellular and cellular

TABLE VI

Summary of deviation from control values of exchangeable sodium, exchangeable chloride, and chloride^{36} space

Experiment no.	Average % deviation from control		
	Exchangeable Na	Exchangeable Cl	$V_{Cl^{36}}$
Respiratory acidosis			
1 Ac	+2.6	−2.0	+1.7
2 Ac	+3.1	+2.6	+5.0
3 Ac	+2.3	+0.5	+0.9
4 Ac	—	+1.7	+4.2
14 Alk-Ac	−2.5	+5.6	+7.3
8 A	−1.9	+0.7	+3.3
Respiratory alkalosis			
12 Alk	0.0	+1.7	−1.5
14 Alk-Ac	−1.9	+1.8	+2.0

or supportive structures in respiratory acidosis and alkalosis are independent of the nature of the substance employed in measuring extracellular fluid volume. Quantitatively, the magnitude of the shifts observed are of course dependent on the volume measured and thus on the nature of the substance employed. We have chosen the volume of distribution of radiosulfate as a measure of readily available extracellular fluid on the evidence of Swan, Madisso, and Pitts (20). We define readily available extracellular fluid as plasma and that fraction of the interstitial fluid with which it is in ready diffusion equilibrium.

Following nephrectomy, neither the measured volume of extracellular fluid (20), nor its electrolyte content, changes significantly over several hours (32). As shown above, the volume of distribution of radiosulfate remained unchanged in consequence of the induction of either severe respiratory acidosis or alkalosis. That extracellular fluid volume remained unchanged is further supported by the observation that the volumes of distribution of sucrose and radiochloride likewise remained unaltered. Possible errors in estimating extracellular fluid electrolyte content include changes of Gibbs-Donnan distribution factor with changes in pH, and changes in the readily avail-

able extracellular fluid volume by water transfers (33, 34) not detected by the methods used. In our experiments, the calculated transfer of water into red cells in respiratory acidosis, based on five observations, reveals a shift of the magnitude of about 2.0 per cent of the total ECF, a value well within the limits of error of estimating the volume of distribution of radiosulfate. In three alkalosis experiments the water shift out of red cells was similarly only 1.5 per cent of the measured extracellular fluid volume. These changes are believed to be too small to affect the conclusions drawn.

The data presented indicate that significant redistribution of electrolytes occurs in respiratory acidosis and alkalosis in the absence of renal compensatory mechanisms. In acute respiratory acidosis, the decrease of fixed acid and the increase of base in the extracellular fluid permit the expansion of buffer anions to a degree which diminishes considerably the severity of the developing acidosis. Similarly, in acute respiratory alkalosis of the severity induced in these experiments, compensatory reduction of buffer anions in the extracellular fluid occurred, thereby preventing the development of a lethal alkalosis.[8]

To depict the participation of these mechanisms in the changes of buffer content observed, Figures 6 and 7 are presented. These graphs are based on observations limited to those experiments in which all the respective ions have been determined. Since a fair degree of variation occurs, they indicate general trends of responses to acute respiratory acidosis and alkalosis. In Figure 6 the total increment of buffer anions of the extracellular

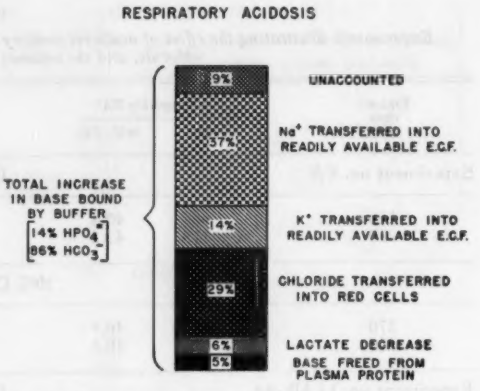

FIG. 6. THE MODE OF EXTRACELLULAR BUFFERING IN ACUTE RESPIRATORY ACIDOSIS

Data from four experiments are averaged (9 Alk–Ac, 11 Ac–Alk, 13 Alk–Ac, 14 Alk–Ac). Gas mixtures used were in all experiments 20 per cent CO_2 to 80 per cent O_2. In Figures 6 and 7 $HPO_4^=$ was calculated using the Henderson-Hasselbalch equation assuming a pK′ for phosphate of 6.8.

fluid during acute respiratory acidosis is represented by the total height of the column. The largest fraction of this increment in buffer anions is due to the increase of bicarbonate and only to a slight extent to the increment in basic phosphate ($HPO_4^=$). The contributions of the various ion species to the total increase are expressed as percentages.

Only a small portion of the total increase is mediated by a decrease in base binding of plasma proteins (35) as consequence of the pH fall (5 per cent) and by decrease of the fixed acid anion lactate (6 per cent). As can be seen, the major portion of buffer anion increase is made possible by fixed acid chloride leaving the extracellular fluid (29 per cent) and base being transferred into it. The total cation increment is made up of sodium (37 per cent) and potassium (14 per cent). The fraction unaccounted for (9 per cent) probably represents changes in undetermined constituents, such as organic acids, calcium, and magnesium.

Figure 7 is a similar representation of changes observed in acute respiratory alkalosis. In this instance the total height of the column indicates the degree of buffer anion decrement in the extracellular fluid. Again, only a very small part is

[8] In the case of respiratory acidosis, induced by breathing a 20 per cent CO_2 mixture, the H_2CO_3 concentration of plasma would rise theoretically to about 5 mM per L. If $[BHCO_3]$ remained unchanged, then the normal ratio $[BHCO_3]/[H_2CO_3]$ of 20:1 would change to 4:1, resulting in a pH of 6.70. In most of our experiments, pH ranged about 6.88, indicating an alteration of this ratio to 6:1. Thus extrarenal compensation effected an increase in $[BHCO_3]$ of approximately 10 mM per L. of plasma. A similar analysis of the situation in acute respiratory alkalosis of the degree achieved in our experiments, where $[H_2CO_3]$ falls to approximately 0.2 mM per L., reveals that were no compensatory displacement of $[BHCO_3]$ to occur, a pH of 8.1 would result. That the pH rises only to approximately 7.7 indicates that extrarenal compensation has permitted a fall of $[BHCO_3]$ of about 12 mM per L. plasma.

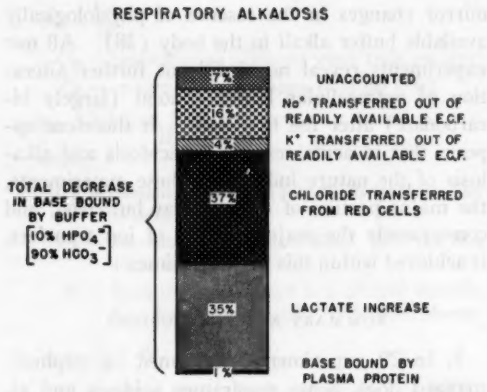

FIG. 7. THE MODE OF EXTRACELLULAR BUFFERING IN ACUTE RESPIRATORY ALKALOSIS

Data from five experiments are averaged (9 Alk–Ac, 11Ac–Alk, 12 Alk, 13 Alk–Ac, 14 Alk–Ac). The pCO_2 in these experiments uniformly fell to levels of 6 to 8 mm. Hg.

due to altered base binding of plasma proteinates (1 per cent). However, the increase in lactate markedly contributes to the displacement of buffer anion (35 per cent). Further displacement of buffer anion is effected by transfer of chloride into the extracellular fluid (37 per cent) and disappearance of sodium (16 per cent) and potassium (4 per cent) from it. The remaining undetermined 7 per cent may include changes in various other anions or cations, such as keto-acids and serum calcium (36).

In the following an attempt is made to analyze in somewhat more detail the pertinent changes in fixed acid and base which occur in the nephrectomized animal during respiratory acid-base disturbances.

Changes in blood *lactate* levels have been observed repeatedly in alkalosis of respiratory and metabolic origin (11, 37, 38). So far, the precise etiology of these alterations is not known, but several factors may be involved in its origin. Peters and Van Slyke (38) have suggested that in an alkaline medium, hemoglobin may deliver oxygen to the tissues less efficiently because of the shift to the left of its oxygen dissociation curve (39), thus favoring anaerobic glycolysis. Similarly, circulatory stasis, related to lowering of blood pressure (36) and peripheral vasoconstriction (40), both of which are known to occur in respiratory alkalosis, may lead to lack of available oxygen.

A somewhat different view is presented by Katzman, Villee, and Beecher (41). *In vitro* studies of tissue slices in an adequately oxygenated environment indicate that with bicarbonate concentration of the system kept constant, lactate production varies inversely with pCO_2. This may be interpreted to reflect a direct effect of carbon dioxide partial pressure on lactate metabolism. A further factor, suggested by several authors (42) has been increased epinephrine secretion. The markedly increased lactate levels observed in respiratory alkalosis are probably the result of a combination of the above factors, all of which favor anaerobic glycolysis. Our experiments, in which both alkalosis and acidosis were induced, indicate that these alterations in lactate metabolism are readily reversible.

Regarding the *chloride* changes observed, it has been found in animals with intact kidneys that total plasma anion concentration ($Cl + HCO_3$) is maintained in the presence of marked alterations of anion pattern (43). Thus, an inverse relationship between plasma chloride and bicarbonate has been observed in respiratory acid-base disturbances (44). Our experiments show that this condition holds in the arenal animal as well. Therefore, these changes must be due to transfers of chloride and bicarbonate across an extracellular boundary. Our experimental results indicate that the chloride shift across the red cell membrane, as classically described for whole blood by various authors (45, 46), appears of sufficient magnitude to account for all the chloride which leaves the extracellular fluid in respiratory acidosis, or conversely, enters it in respiratory alkalosis. In this manner the significant buffer capacity of the red cell, largely in the form of hemoglobin, is made available to the extracellular fluid. In some experiments, however, the chloride shift to or from the extracellular fluid seemed larger than could be accounted for by a red cell transfer alone. The possibility remains, therefore, that chloride shifts may occur across some other extracellular boundary. Our data certainly indicate that a significant fraction of exchangeable chloride does exist outside of the readily available extracellular space.

Alteration of extracellular *base* content was a regular finding in the studies reported. In respiratory acidosis a discharge of sodium and potassium into the readily available extracellular space occurred consistently. These moieties presumably were derived from cells or supportive structures. Our data indicate that the red cells are not responsible for the cation transfers observed. Conversely, extracellular cations must have entered cells or/and supportive structures in respiratory alkalosis. It seems likely that sodium and potassium were exchanged mole for mole for hydrogen ions in this process (10). The transfers of sodium occurred without alteration in the size of the exchangeable sodium pool as measured by radiosodium. The store of exchangeable bone sodium is potentially large (47). Evidence indicates that this reserve of base may be called upon in the maintenance of extracellular ion composition (48, 49) in acid-base disturbances. Under the acute conditions of our experiments, exchanges apparently represent redistribution of sodium without mobilization of previously non-exchangeable sodium. Balance studies conducted in human subjects by Singer, Elkinton, Barker, and Clark (50) (based on the chloride space), have revealed shifts of cell base in acute respiratory acidosis and alkalosis comparable to those reported here.

Significant changes in plasma inorganic *phosphate* levels were in accord with older observations of Haldane, Wigglesworth, and Woodrow (51) who found a significant rise in respiratory acidosis, and a fall in alkalosis. Similarly, Fitz, Alsberg, and Henderson (52) and Goto (5) showed earlier that the large intracellular stores of organic phosphate may be drawn upon in furnishing extracellular buffer anion. On the other hand, an increase in intraerythrocytic organic acid soluble phosphorus has been demonstrated in metabolic alkalosis (53). The etiology of these changes has been studied by Mackler and Guest (54). These authors observed that acidosis inhibits phosphorylation of glucose, both *in vitro* and *in vivo*. In this connection, the older finding of Lawaczeck (55), which showed that an increase of hydrogen ion concentration, induced by an increase in pCO_2, accelerates hydrolysis of red cell organic phosphates, may be pertinent.

Bicarbonate is the most important of the extracellular buffer anions; alterations of its content mirror changes in the reserve of physiologically available buffer alkali in the body (38). All our experiments reveal no significant further alteration of extracellular buffer content (largely bicarbonate) after the first hour. It therefore appears that in acute respiratory acidosis and alkalosis of the nature induced in these experiments, the major portion of extracellular buffering, and consequently the major portion of ion transfers, is achieved within this period of time.

SUMMARY AND CONCLUSIONS

1. In 29 experiments performed on nephrectomized dogs, acute respiratory acidosis and alkalosis were induced.

2. Volumes of distribution of radiosulfate, of sucrose, and of radiochloride did not change. From these findings, it is concluded that extracellular fluid volume remained unaltered.

3. Significant redistribution of electrolytes across the boundary of the readily available extracellular fluid volume was observed.

4. Tissue contribution to buffering within the extracellular fluid was found to be mediated by the following mechanisms:

a) Transfer of chloride across the erythrocyte membrane;
b) shift of sodium and potassium across an extracellular boundary other than the red cell membrane;
c) transfer of inorganic phosphate across an unknown extracellular boundary;
d) significant alterations in lactate metabolism.

ACKNOWLEDGMENTS

We would like to express our appreciation to Drs. H. D. Lauson and R. C. Swan for helpful advice.

APPENDIX

The following symbols and calculations were used to derive data on electrolyte content of extracellular fluid and red cells:

1) Water correction, when used, was calculated as:

$$W = 1 - \frac{P_s}{100}$$

where

W = plasma water correction factor

P_s = plasma proteins in grams per 100 ml. plasma

2) $C_W = \frac{C_P}{W}$

C_W = concentration in plasma water (mM per liter)

C_P = concentration in plasma (mM per liter)

3) $C_R = \frac{C_B - (1 - H)\, C_P}{H}$

where

C_R = concentration in red blood cells (mM per liter of cells)

C_B = concentration in whole blood (mM per liter whole blood)

H = Hematocrit, expressed as a decimal fraction. No correction was applied for trapped plasma.

4) $V_S = \frac{I_S}{C_0} \times W$

V_S = volume of distribution of sucrose (ml. H_2O)

I_S = amount of sucrose injected (mg.)

C_0 = plasma concentration of sucrose at zero time (mg. per ml.)

C_0 was obtained by extrapolating to zero time a straight line which could be fitted by eye to the values of the logarithm of successive plasma concentrations plotted against time.

5) The volume of distribution of radiosulfate was estimated by a slightly modified formula, suggested by Gamble and Robertson (56).

$$V_{S^{35}O_4} = W \cdot PV + \frac{W}{D}\left[\frac{I_{S^{35}O_4}}{C_0} - PV\right]$$

$V_{S^{35}O_4}$ = volume of distribution of $S^{35}O_4$ (ml. H_2O)

PV = plasma volume (ml. plasma)

D = Donnan factor for $S^{35}O_4$, taken as 1.1 (20)

$I_{S^{35}O_4}$ = amount of $S^{35}O_4$ injected (counts per min.)

C_0 = concentration of $S^{35}O_4$ at zero time, in counts per minute per ml. plasma. C_0 was obtained in a manner similar to that described for C_0 of sucrose.

6) $V_{Cl^{36}} = W \cdot PV + \frac{W}{D}\left[\frac{I_{Cl^{36}}}{C_t} - PV\right]$

Volume of distribution of Cl^{36} was determined by Formula 5, save that D = 1.05, and C_t is taken as the concentration of Cl^{36} in counts per min. per ml. plasma at the hourly intervals.

7) Plasma volume was determined once in control periods and once every two to three hours thereafter.

$PV_C = PV \cdot W$

PV_C = volume of distribution of T-1824 (taken as estimate of plasma volume) in ml. H_2O

PV = plasma volume in ml. plasma

8) Total amounts of electrolytes in the readily available extracellular fluid (ECF) were obtained by use of data of plasma concentration, plasma volume, interstitial fluid volume, and appropriate Donnan and water correction factors. The following formula was used:

$$T_E = \frac{PV \cdot C_P}{1000} + [V_{S^{35}O_4} - PV_C]\frac{C_W \cdot D}{1000}$$

T_E = total mM in $V_{S^{35}O_4}$

D = Donnan factor, 0.95 for Na and K. 1.05 for Cl and HCO_3.

No Donnan factor was used for PO_4 and lactate.

9) Total amounts of electrolytes in the total red blood cell volume were calculated by use of data on red blood cell concentration, plasma volume, and hematocrit. No correction for excess plasma or excess red cells was applied because of the findings of Reeve, Gregersen, Allen, and Sear (30) that the ratio of total body hematocrit to peripheral hematocrit varied from 0.9 to 1.1 in dogs under different experimental conditions. The following formula was used to calculate total electrolytes in red cell volume:

$$T_C = \frac{C_R}{1000}\left[\frac{PV}{1 - H} - PV\right]$$

T_C = total red cell electrolytes in mM.

10) Base binding of plasma proteinate (BP_B in mEq. per L.) was estimated according to Singer and Hastings (35).

11) Exchangeable sodium and chloride were estimated in the usual way (57).

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PHYSIOLOGICAL AND PHARMACOLOGICAL REGULATION OF HUMAN SALIVARY ELECTROLYTE CONCENTRATIONS; WITH A DISCUSSION OF ELECTROLYTE CONCENTRATIONS OF SOME OTHER EXOCRINE SECRETIONS [1]

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The electrolyte content of paraffin-stimulated, whole, mixed saliva obtained from patients with congestive heart failure is characterized by lower sodium, lower chloride, and higher potassium concentrations than saliva of normal subjects (1). Since these data suggested possible increased adrenal cortical activity in patients with heart failure, desoxycorticosterone acetate (DCA) was administered to nineteen normal subjects. It also seemed of interest to study additional pharmacological and physiological mechanisms that may play a role in the regulation of salivary electrolyte concentrations. Thus, the aims of this communication are to present: 1) Data on normal salivary concentrations of sodium, chloride, and potassium; 2) data relating to physiological mechanisms regulating the concentrations of salivary electrolytes; and 3) the effects on salivary electrolytes of three pharmacologic agents: desoxycorticosterone acetate (DCA), pilocarpine, and Mercuhydrin®.

METHODS

Approximately 30 ml. of saliva were collected in the morning from fasting subjects who chewed paraffin for 15 to 20 minutes following a preliminary mouth wash with water. Where indicated, normal subjects chewed paraffin for two consecutive ten-minute periods. Whole, mixed saliva was analyzed. Sodium and potassium concentrations were measured with an internal standard Perkin 52A flame photometer; chloride concentrations, by the Van Slyke-Hiller iodometric method (2). Standard methods of statistical analysis were employed (3); the "t" test was used to determine the statistical significance of mean differences between groups, and the method of "paired differences" was employed in evaluating the effects of pharmacologic agents on the electrolyte concentrations of saliva.

Ten mgm. DCA in oil were injected intramuscularly, daily, for 5 to 15 days into normal subjects who received a regular diet and 10 gm. supplemental salt by mouth per day. Duplicate collections of saliva were collected on consecutive days both before and during the administration of DCA in order to test the reproducibility of the values for salivary electrolyte concentrations. The concentration of potassium was not measured in one of these subjects.

Twenty-nine pairs of observations were made on the effect of 6 mgm. of subcutaneously-administered pilocarpine on the salivary electrolyte concentrations in subjects without cardiac disease. Thirty minutes after the injection of pilocarpine the subject began to chew paraffin again, and 30 ml. of saliva were collected (usually in about 10 minutes).

Two ml. of Mercuhydrin® were injected intramuscularly into 10 patients with congestive heart failure and on a low salt diet. Collections of saliva were made at the time of administration, and at 1½, 3, and 24 hours, respectively, after the injection of the mercurial diuretic.

Fifteen experiments on nine normal subjects on a regular salt diet were performed in which saliva was collected in two successive 10-minute periods in order to compare the salivary electrolyte concentrations during varying rates of flow.

RESULTS

Normal concentrations of salivary electrolytes

For the 73 control subjects on a regular salt diet, including the 16 values obtained from our earlier studies (1), the normal salivary concentrations of sodium, chloride, and potassium are 26.4 ± 11.8, 29.0 ± 8.8, and 19.7 ± 3.9 mEq. per L., respectively.

[1] Aided by a grant from the American Heart Association and by a United States Public Health Service grant (H-1245).

[2] This work was begun during the tenure of a Research Fellowship of the American Heart Association in 1950. Present address: The Mount Sinai Hospital, New York City.

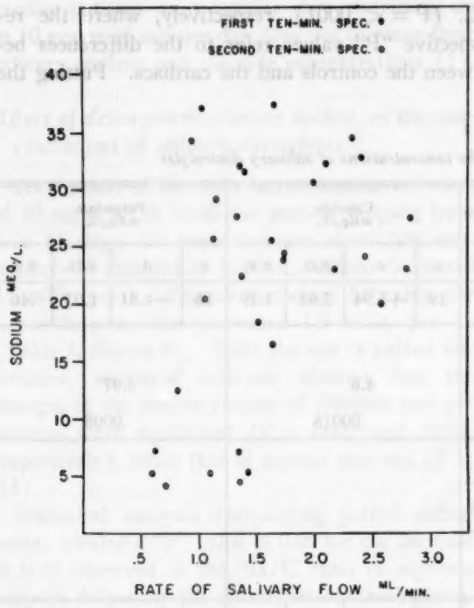

FIG. 1. RELATION OF THE SALIVARY CONCENTRATION OF SODIUM (MEQ. PER L.) TO RATE OF SALIVARY FLOW (ML. PER MIN.)

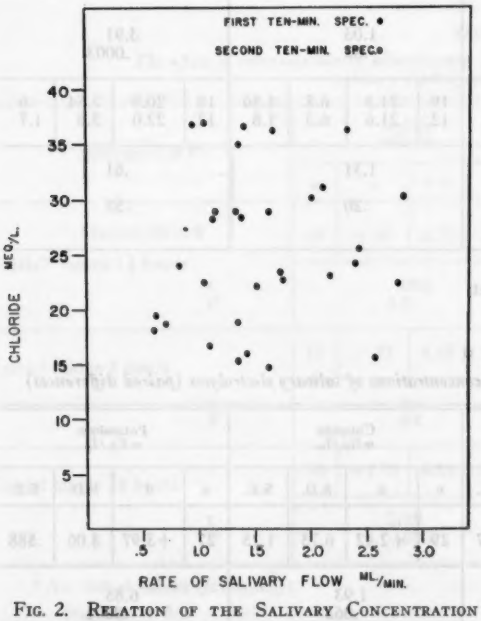

FIG. 2. RELATION OF THE SALIVARY CONCENTRATION OF CHLORIDE (MEQ. PER L.) TO RATE OF SALIVARY FLOW (ML. PER MIN.)

Effect of rate of flow on the concentrations of salivary electrolytes

There does not appear to be any highly significant effect of the rate of flow on these values (Figures 1, 2, 3). For the first 10-minute specimens, the regression lines, together with the corresponding correlation coefficients (r) are:

Na: $Y_e = 14.83 + 5.98 \times (r = .433)$
Cl: $Y_e = 27.1 + 1.22 \times (r = .283)$
K: $Y_e = 21.2 - 0.8 \times (r = .283)$

The regression lines for the second 10-minute specimens are:

Na: $Y_e = 9.3 + 6.15 \times (r = .5)$
Cl: $Y_e = 22.7 - 0.45 \times (r = .265)$
K: $Y_e = 24.5 - 2.68 \times (r = .51)$

Combined values for control subjects and for patients with congestive heart failure

The salivary concentrations of sodium, chloride, and potassium in the present series of patients with congestive heart failure are essentially the same as those for our earlier cardiac series. There is a statistically significant difference in the salivary electrolyte concentrations between the combined control group and the combined series of patients with congestive heart failure; for the

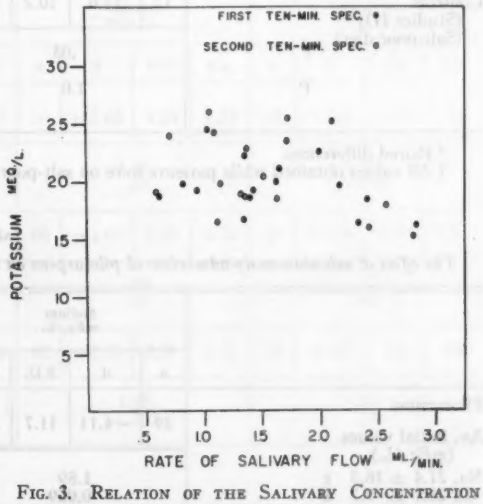

FIG. 3. RELATION OF THE SALIVARY CONCENTRATION OF POTASSIUM (MEQ. PER L.) TO RATE OF SALIVARY FLOW (ML. PER MIN.)

latter, the salivary concentrations of sodium, chloride, and potassium are 17.8 ± 8.9 (P = < .0001), 22.2 ± 8.4 (P = .0001), and 24.7 ± 4.8 mEq. per L. (P = < .0001), respectively, where the respective "P" values refer to the differences between the controls and the cardiacs. Putting the

TABLE I

The effect of desoxycorticosterone acetate on the concentrations of salivary electrolytes

Subjects	Sodium mEq./L.				Chloride mEq./L.				Potassium mEq./L.			
	n	d	S.D.	S.E.	n	d	S.D.	S.E.	n	d	S.D.	S.E.
Pre-DCA minus DCA*	19	+3.79	10.8	2.47	19	+5.94	5.63	1.29	18	−1.81	1.93	.46
Av. initial values (*mEq./L.*)												
Na, 21.3 ± 15.0 t		1.53				4.6				3.97		
Cl, 30.2 ± 9.0												
K, 19.1 ± 2.4 P		.14				.00018				.0008		
Na/K ratio,	Na/K											
Pre-DCA minus DCA*	18	+.245	.585	.138								
t		1.77										
P		.09										
	n	x	S.D.	S.E.	n	x	S.D.	S.E.	n	x	S.D.	S.E.
DCA	19	17.5	10.9	2.5	19	24.3	6.8	1.56	18	20.9	2.54	.6
Combined CHF†	39	17.8	8.9	1.43	39	22.2	8.4	1.33	39	24.7	4.8	.76
(Salt-poor diet)												
t		.104				1.03				3.91		
		.92				.32				.0003		
DCA	19	17.5	10.9	2.5	19	24.3	6.8	1.56	18	20.9	2.54	.6
Controls	12	17.6	10.2	3.0	12	21.6	6.3	1.8	12	22.0	5.8	1.7
(Studies II)†												
(Salt-poor diet)												
t		.03				1.31				.61		
P		1.0				.20				.55		

* Paired differences.
† All values obtained while patients were on salt-poor diet.

TABLE II

The effect of subcutaneously-administered pilocarpine on the concentrations of salivary electrolytes (paired differences)

	Sodium mEq./L.				Chloride mEq./L.				Potassium mEq./L.			
	n	d	S.D.	S.E.	n	d	S.D.	S.E.	n	d	S.D.	S.E.
Pilocarpine	29	−4.11	11.7	2.17	29	+2.42	6.75	1.25	27	+3.97	3.06	.588
Av. initial values (*mEq./L.*)												
Na, 27.4 ± 16.3 t		1.89				1.93				6.85		
Cl, 26.7 ± 7.7 P		0.069				.065				.0001		
K, 20.5 ± 2.8												

cardiac patients on a regular diet containing up to 10 grams of salt per day does not increase their salivary sodium and chloride concentrations (1).

Effect of desoxycorticosterone acetate on the concentrations of salivary electrolytes

As a result of the daily intramuscular injection of 10 mgm. DCA in oil for periods ranging from 5 to 15 days, the mean salivary electrolyte concentrations changed as follows: sodium decreased 3.8 mEq. per L., chloride decreased 5.9 mEq. per L. while potassium increased 1.8 mEq. per L. (Table I, Figure 4). With the use of paired differences, statistical analysis showed that the changes in the concentrations of chloride and potassium were significant (P = .0002 and .0008, respectively), while that of sodium was not (P = .14).

Statistical analysis (employing paired differences) yielded a "P" value of 0.09 for the decrease of 0.25 observed in the Na/K ratio in eighteen subjects following the intramuscular administration of desoxycorticosterone acetate. No measurements were made of the effects of DCA on the rates of flow, but no gross changes were evident.

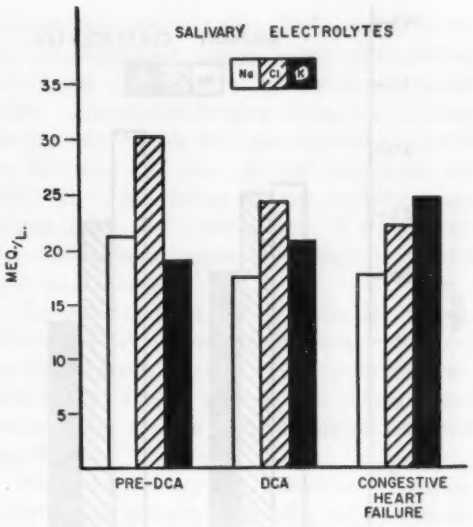

FIG. 4. EFFECT OF INTRAMUSCULARLY-ADMINISTERED DESOXYCORTICOSTERONE ACETATE (DCA) ON SALIVARY CONCENTRATIONS OF SODIUM, CHLORIDE, AND POTASSIUM (MEQ. PER L.)

The corresponding concentrations in patients with congestive heart failure are shown for comparison.

TABLE III

The effect of intramuscularly administered Mercuhydrin® (2 ml.) on the concentration of salivary electrolytes (paired differences)

Subjects—C.H.F.	Sodium mEq./L.				Chloride mEq./L.				Potassium mEq./L.			
	n	d	S.D.	S.E.	n	d	S.D.	S.E.	n	d	S.D.	S.E.
Mercuhydrin® Initial* minus 1½ hours	10	+.01	6.55	2.07	10	−2.65	4.29	1.35	10	−1.4	2.24	.71
t	.005				1.96				1.97			
P	1.0				.08				.095			
Initial minus 3 hours	10	−.87	6.26	1.98	10	−1.63	5.46	1.73	10	−1.39	6.02	1.9
t	.44				.94				.73			
P	.68				.35				.48			
Initial minus 24 hours	10	+2.98	4.31	1.36	10	+2.27	5.97	1.91	10	+.27	3.1	.98
t	2.19				1.19				.28			
P	.056				.26				.78			

* Av. initial values (mEq./L.):

Na, 15.4 ± 8.2
Cl, 22.5 ± 7.7
K, 23.8 ± 5.2

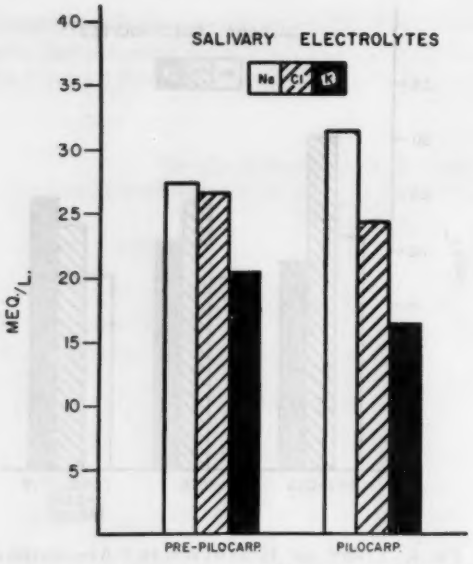

FIG. 5. THE EFFECT OF SUBCUTANEOUSLY-ADMINISTERED PILOCARPINE ON THE SALIVARY CONCENTRATIONS OF SODIUM, CHLORIDE, AND POTASSIUM

Effect of pilocarpine on concentrations of salivary electrolytes

Pilocarpine (6 mgm. administered subcutaneously) lowered the salivary concentration of potassium 3.97 mEq. per L. ($P = 0.0001$), lowered the chloride 2.42 mEq. per L. ($P = .065$), and increased the sodium 4.11 mEq. per L. ($P = .069$) (Table II, Figure 5). No measurements were made of the rates of flow in this group of experiments.

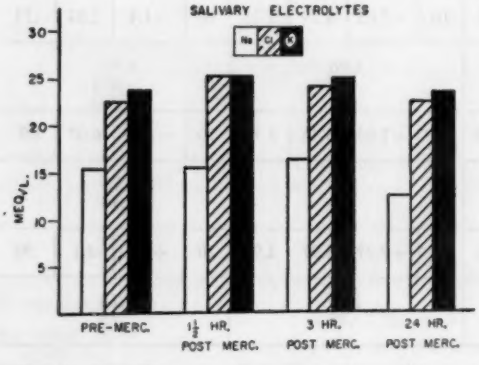

FIG. 6. THE EFFECT OF INTRAMUSCULARLY-ADMINISTERED MERCUHYDRIN® ON THE SALIVARY CONCENTRATIONS OF SODIUM, CHLORIDE, AND POTASSIUM

Effect of Mercuhydrin® on concentrations of salivary electrolytes

Mercuhydrin® (2 ml. injected intramuscularly) had no statistically significant effect on the salivary concentrations of sodium, chloride, or potassium, 1½, 3, and 24 hours following the injection of the diuretic (Table III, Figure 6). No measurements were made of the rates of flow.

DISCUSSION

Normal concentrations of salivary electrolytes

A. *Methods of collecting saliva.* Babkin (4) has summarized the basic physiological mechanisms underlying salivary secretion. Several important facts emerge regarding variations in the concentrations of electrolytes in human, mixed saliva. The nature of salivary secretion depends upon the type and duration of stimulus, and there also may be differences in the composition of saliva obtained from the parotid gland, on the one hand, and the mixed glands, on the other. Furthermore, it seems probable that there is no such thing as un-stimulated saliva in the human, because as soon as the subject opens his mouth, the oral cavity (and salivary glands) may receive secretory stimuli such as air currents. Therefore, the flow and composition of whole, mixed, human saliva represent a net result of multiple factors. It follows that almost any method of collecting human saliva must be somewhat arbitrary, but may suffice if: a) It yields a reproducible salivary composition; and b) variations in composition following an experimental stimulus or pharmacologic agent are not inhibited by the nature of the collecting method. The methods presented appear to satisfy these two requirements.

B. *Effect of rate of salivary flow on the concentrations of electrolytes.* Although the data presented are somewhat limited, they appear to indicate that under these experimental conditions, variations in the rate of human, mixed, salivary flow do not have any statistically, highly significant effects on the *concentrations* of sodium, chloride, or potassium (highest "r" value calculated equals 0.51). Obviously, if the concentrations do

not vary significantly with the rate of flow, there will tend to be a linear relationship between the rate of excretion of an electrolyte (mEq. per min.) and the rate of flow (ml. per min.).

In contrast to these results, Brown and Klotz (5) found that following the chewing of paraffin for one hour by eighteen subjects, the salivary concentrations of chloride and sodium varied directly with the rate of flow, while that of potassium tended to decrease slightly with increase in the rate of secretion. The much longer duration of stimulus to salivary secretion and the higher rates of salivary flow in their experiments may account for the discrepancy in the findings.

Studying the secretion of saliva by the dog's submaxillary gland following stimulation of the chorda tympani or the administration of pilocarpine, Gregersen and Ingalls (6) found that the concentration of sodium varied between 10 and 90 mEq. per L., depending closely on the rate of secretion. The concentration of potassium, on the other hand, remained approximately constant at 10 to 20 mEq. per L., depending on the type of stimulus (compare with the average of 19.7 ± 3.9 mEq. per L. for 73 human subjects in the present series). Not only must the species difference be considered in comparing our findings with those of Gregersen and Ingalls, but their collection of saliva from one gland, the submaxillary, while in the present studies mixed saliva was collected (at a slower rate of flow) from all of the salivary glands. Furthermore, the stimulus was different, and Baxter (7) has reported that the concentrations of chloride, calcium, and potassium were much lower in the parotid saliva of dogs following the administration of pilocarpine than in the parotid saliva secreted *at the same rate* following the feeding of meat extract or bread and meat powder. It is apparent that the effect of the rate of salivary secretion on the concentrations of electrolytes is determined in part by the duration and type of stimulus.

Effect of desoxycorticosterone acetate (DCA) on the salivary concentrations of electrolytes

Since these studies had their origin in earlier observations (1) suggesting adrenocortical control of salivary electrolyte concentrations, it is of interest that treatment with DCA resulted in statistically significant changes in the concentrations of chloride and potassium but not in that of sodium. Dissociation between changes in salivary sodium and chloride has been reported previously by McCance (8) who observed that acute salt deficiency in five normal subjects produced a consistent fall in the concentrations of salivary sodium while the corresponding changes in chloride were inconsistent.

The mean decrease of 0.25 in the Na/K ratio following DCA is similar to the decrease observed by Frawley and Thorn (9) 24 hours after administering 15 mgm. DCA to each of two normal subjects, but appears to be of limited statistical significance.

While the salivary concentrations of sodium, chloride, and potassium following the administration of DCA approached those reported previously for normal subjects on a low-salt diet (1), the post-DCA values for salivary potassium did not rise to the relatively high levels observed in our patients with congestive heart failure. A possible explanation is that the imposition of a low-salt regimen does not stimulate the elaboration of endogenous adrenocortical hormones in entirely the same way as the onset of congestive heart failure, so that a low-salt diet may stimulate the elaboration and secretion of greater amounts of a desoxycorticosterone-like fraction than does congestive heart failure.

Other work suggesting the adrenocortical regulation of salivary electrolytes includes the observations of Frawley and Thorn (9) on the increased salivary Na/K ratio in patients with Addison's disease, with a decrease observed in Cushing's syndrome. These investigators also reported a fall of 18 to 68 per cent in the salivary Na/K ratio following a standard 48-hour ACTH test administered to eight normal subjects, while under the same circumstances, four patients with Addison's disease showed no fall. Grad (10) has also reported a fall in the salivary Na/K ratio following the administration of ACTH to four subjects, while Dreizen, Niedermeier, Reed, and Spies (11) reported decreases in salivary sodium following intramuscularly-administered ACTH or cortisone but no change in potassium.

Effect of pilocarpine on the concentrations of salivary electrolytes

Previous studies on animals have demonstrated both increases and decreases in the salivary concentrations of sodium and chloride following the administration of pilocarpine, but all have been in agreement on the resulting decrease in the concentration of potassium. Evidence that this decrease in potassium cannot be ascribed solely to changes in the rate of salivary flow is provided by the work of Gregersen and Ingalls (6). Employing either electrical stimulation of the chorda tympani or the administration of pilocarpine, they demonstrated that for comparable rates of submaxillary salivary flow the concentrations of potassium (following pilocarpine) is approximately one-half that observed after stimulation of the chorda tympani. Following the administration of pilocarpine the potassium content of the submaxillary gland is decreased, while it remains essentially unchanged after electrical stimulation of the chorda tympani (12–14); these observations strengthen the probability that pilocarpine has a specific metabolic effect upon the salivary glands.

Effect of Mercuhydrin® on the salivary concentrations of electrolytes

The absence of any significant effect of Mercuhydrin® on the salivary concentrations of sodium, chloride, and potassium tends to emphasize the localized renal action of mercurial diuretics with respect to effects on the excretion of sodium, chloride, and potassium. In the dog, the intravenous administration of biochloride of mercury had no effect on the concentrations of electrolytes in saliva flowing in response to electrical stimulation of the chorda tympani (15).

Relation of concentrations of electrolytes in saliva and blood, with discussion of possible regulating physiological mechanisms

The salivary concentrations of sodium and chloride are much lower than their corresponding concentrations in serum, while that of potassium is approximately four times the serum concentration of this ion. In support of this lack of correlation between serum and salivary concentrations of sodium chloride, Doneddu (16) observed that the intravenous injection of 20 ml. of 30 per cent sodium chloride into human subjects did not significantly affect the salivary concentration of this salt in any case. De Beer and Wilson (17) studied the concentrations of sodium, chloride and potassium in parotid saliva after pilocarpine in dogs under Amytal® anesthesia. While the concentrations of chloride (87 to 104 mEq. per L.) and sodium (123 to 130 mEq. per L.) tended to approach those in the serum at high rates of flow, the concentration of potassium (10.3 to 12.3 mEq. per L.) was higher than that in serum.

The classic explanation for the lower concentrations of sodium and chloride in saliva than in plasma has postulated an impaired permeability of the salivary glandular cell membrane to these ions (4). Presumably, with increased secretory activity the permeability to movement of sodium and chloride from the cell into the lumen increases, resulting in higher salivary sodium and chloride concentrations.

Possible reabsorptive function of rodded epithelium in excretory ducts. Another possibility, which seems more likely to us, is that sodium and chloride are secreted into the salivary duct lumen in isotonic concentrations and then undergo partial reabsorption in the duct cells. A possible basis for the reabsorption of chloride and/or sodium is provided by a study of the lining cells of the excretory ducts of five exocrine glands (the three salivary glands, the lacrimal gland, and the pancreas) Table IV. In 1883, Merkel (18) described the distribution of the rodded epithelial cells (Streifenzellen) in the excretory-duct systems of the salivary glands, pointing out that while the sublingual salivary gland has no such cells, the submaxillary has the longest tract of these cells and the parotid, a fairly well-developed duct system lined by rodded epithelium. In addition, the lacrimal glands and the pancreas, like the sublingual salivary gland, have no rodded epithelium in their excretory ducts. He postulated that the function of these cells is one of secretion of water and salts, especially calcium. However, the observed ash content of the secretions of the three glands does not agree with the amounts to be expected on the basis of Merkel's hypothesis; for example, the sublingual saliva has the highest ash content instead of the lowest, as would be

TABLE IV

The relation between the presence of "rodded epithelium" in the ducts of exocrine glands and electrolyte concentrations of their corresponding secretions

Gland	"Rodded epithelium" in duct (4)*	Secretion			
		Relative ash content (19)	Absolute ash content (20)	Cl *mEq./L.*	Na *mEq./L.*
Salivary Sublingual	Practically absent	2.0		121–185 (19) 143 (20)	
Parotid	Well developed	1.0	0.77%	13.3–40.9 (19)	
Submaxillary	Longest	0.7	0.53%	25.6–57.3 (19) 51.3 (20)	
Lacrimal	Absent			154 (21)	154 (21)
Pancreas	Absent				150 (22)
Mammary	Not known			9–34 (25)	5–26 (25)
Sweat	Not known			15–60 (26)	15–60 (26)

* Reference numbers within parentheses.

in keeping with the absence of secretory, rodded epithelium.

Further evidence against the secretory nature (with respect to electrolytes) of the rodded epithelial cells was provided in 1886 by Werther (19) who employed pilocarpine stimulation, and confirmed much later by Baxter (20), who used stimulation of the chorda tympani. Both of these authors measured the chloride concentrations of saliva obtained separately from each of the three salivary glands. The sublingual saliva has the highest concentration of chloride—even higher than that of plasma—while the parotid and submaxillary glands yielded saliva with chlorides ranging from 13.3 to 57.3 mEq. per L. (Table IV).

The sodium and chloride content of tears is 154 mEq. per L. (21); while the sodium content of the pancreatic secretion is 150 mEq. per L. (22); and as mentioned previously, since no rodded epithelial cells occur in these glands, the concentrations of these ions should be low or nil if these cells indeed secreted electrolytes.

With iron hematoxylin stain rodded epithelial cells can be demonstrated in both the proximal and distal convoluted tubules (23, 24) of the kidney—acknowledged sites of sodium and chloride reabsorption. The concentrations of sodium and chloride in both milk (25), and sweat (26) are hypotonic, and we should expect, therefore, to find cells similar to the rodded epithelial cells in the excretory ducts of these glands. Unfortunately, a review of the available literature has not revealed any detailed histological investigations with the fixing and stains necessary to demonstrate the rodded epithelial cells.

In summary, it is apparent that the concentrations of chloride and/or sodium are isotonic with the plasma or even somewhat hypertonic in the secretions of those exocrine glands having no rodded epithelial cells in their excretory ducts. On the other hand, the chloride and/or sodium concentrations are hypotonic to plasma in the secretions of those glands having rodded epithelial cells in their excretory duct systems. This suggests, to us, a reabsorptive function of these cells.

Secretion of potassium. The high salivary concentration of potassium probably signifies a process of active secretion of this ion by the gland. A process of concentration through duct cell reabsorption of water seems to be excluded by the fact, for example, that the salivary non-protein nitrogen is approximately the same as that of serum.

SUMMARY AND CONCLUSIONS

1. Under standardized conditions of collection, the mean salivary concentration of sodium, potassium, and chloride in 73 normal subjects in the

fasting state after chewing paraffin for 15 to 20 minutes was found to be: Na, 26.4 ± 11.8; Cl, 29.0 ± 8.8; K, 19.7 ± 3.9 mEq. per L.

2. After daily injections of desoxycorticosterone acetate (DCA) in oil for 5 to 15 days into 19 subjects there was a decrease of 3.8 mEq. per L. in sodium ($P = .14$), a decrease of 5.9 mEq. per L. in chloride ($P = .0002$), and an increase in potassium of 1.81 mEq. per L. ($P = .0008$). DCA resulted in a mean decrease of 0.25 in the salivary Na/K ratios ($P = .09$).

3. Following treatment with DCA, the salivary sodium and chloride approached the concentrations of those ions in the saliva of patients with congestive heart failure (Na, 17.8 ± 8.9; Cl, 22.2 ± 8.4 mEq. per L.), but the potassium did not reach the relatively high concentration observed in the cardiac patients (K, 24.7 ± 4.8 mEq. per L.).

4. The salivary concentrations of sodium, chloride, and potassium following DCA treatment were essentially the same as those reported previously by us for normal subjects on a salt-free diet (Na, 17.6 ± 10.2; Cl, 21.6 ± 6.3; K, 22.0 ± 5.8 mEq. per L.).

5. Pilocarpine, 6 mgm. administered subcutaneously to 29 subjects, increased the salivary concentration of sodium by 4.11 mEq. per L. ($P = .065$), and lowered the potassium by 3.97 mEq. per L. ($P = .0001$). Possible mechanisms of action of pilocarpine on the salivary glands are discussed.

6. Two ml. of Mercuhydrin®, administered intramuscularly to 10 patients with congestive heart failure, had no statistically significant effect on the salivary concentrations of sodium, chloride, or potassium, 1½, 3, and 24 hours following the injection of the diuretic.

7. Limited studies, employing the chewing of paraffin as a stimulus, on the effect of the rate of salivary flow on the concentrations of sodium, chloride, and potassium showed some apparent increase in sodium and decrease in potassium as the rate of flow increased. However, these relationships were not as marked as in previous animal experiments reported in which relatively high rates of salivary flow were obtained and other types of stimulation employed.

8. It is suggested that salivary sodium and/or chloride concentrations are hypotonic to their respective serum concentrations as a result of reabsorption of these ions by rodded epithelium in the excretory ducts. A concentration of potassium in saliva higher than that in serum is believed to represent active secretion of this ion by the gland. The concentrations of salivary electrolytes are compared with those in other exocrine secretions in respect to possible regulating mechanisms.

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THE EFFECT OF PHENYLBUTAZONE ON URIC ACID METABOLISM IN TWO NORMAL SUBJECTS

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Phenylbutazone is a potent analgesic compound widely used in the treatment of gout and other arthritic disorders. Both in gouty and non-gouty subjects the drug sharply reduces the serum urate level (1–8). The mechanism by which this is accomplished has recently received attention in several laboratories, but the results are discrepant. Some investigators report that the drug has a potent uricosuric effect (3, 4, 6, 7), others that the drug produces no important change in urate excretion (2, 5), and still others that it actually causes a diminution of urate output (8). The latter two results, if valid, could have several possible explanations, *viz.*, the rate of urate synthesis has been reduced; uric acid has been disposed of by non-urinary pathways, or catabolized, in increased amounts; uric acid has been redistributed throughout a greater percentage of body water; or, uric acid has been greatly diluted in body fluids because of water retention and expansion of extracellular fluid volume. The present study was designed to evaluate these possible metabolic effects of phenylbutazone.

Two normal subjects were placed on constant low purine diets. Uric acid-N^{15} was infused during control periods and again during phenylbutazone administration, the miscible pool of uric acid and the rate of its turnover were determined, and the results obtained were correlated with changes in excretion values and serum concentrations of uric acid. Also, recoveries of unchanged isotopic uric acid in urine were determined in both periods, in order to evaluate extra-renal disposal or catabolism of urates. Finally, calculations of the volumes of distribution of uric acid were made; the changes observed were correlated with body weight and with hematocrit measurements in one subject. These studies have demonstrated that phenylbutazone is a uricosuric drug without specific effect on extra-renal factors of urate metabolism.

METHODS

Subjects. J. W. was a normal white male, 27 years of age, weighing 75 kg. S. N. was a colored female, 52 years of age, weighing 51 kg. She was convalescing from bronchitis and was considered normal at the time of this study. The composition of the diets and the normal urinary urate excretions and serum urate concentrations for these two subjects are given in Table I. Fluid intake was not restricted in either subject.

Isotopic uric acid. Uric acid-1,3-N^{15} having an isotope content of 28.3 atom per cent excess N^{15} was synthesized according to Cavalieri, Blair, and Brown (9) and Wyngaarden and Stetten (10). This product was used in studies on J. W. and a diluted product in studies on S. N.

Plan of experiments. After four days of dietary preparation, continuous 12 or 24-hour urine collections were begun, and frequent samples of serum were obtained in the fasting state for uric acid analyses. After four to five control days, small quantities of uric acid-N^{15} (200 mg. of uric acid, 28.3 atom per cent excess N^{15} in J. W., 86 mg. of uric acid, 6.5 atom per cent excess N^{15} in S. N.) were infused as the lithium salt in 500 ml. saline over a 30-minute period, and uric acid was then isolated from serial 12-hour urine samples for four to six days. Phenylbutazone was subsequently administered orally in dosage of 800 mg. daily, and after a suitable period of time the infusion of uric acid-N^{15} (100 mg. of uric acid, 28.3 atom per cent excess N^{15} in J. W., 68 mg. of uric acid, 6.5 atom per cent excess N^{15} in S. N.) was repeated, and urinary uric acid again isolated. The temporal relationships of these various procedures are self-evident from inspection of Figures 1 and 2.

Analyses.[1] Uric acid was determined in serum and urine according to Praetorius' (11) modification of the differential spectrophotometric method of Kalckar (12) employing purified hog liver uricase prepared according to Muller and Bauer (13). Uric acid was isolated from urine by adsorption onto and elution from charcoal (14) and was recrystallized from Li_2CO_3 solution (15). The miscible pool of uric acid and the rate of its turnover were calculated according to Benedict, Forsham, and Stetten (16). The intercept and slope of the best straight

[1] The author is indebted to Dr. DeWitt Stetten, Jr., of The Department of Health, Education, and Welfare, National Institutes of Health, Bethesda, Maryland, for the N^{15} analyses on subject J. W., and to Dr. Julius White, of the National Cancer Institute, Bethesda, Maryland, for those on subject S. N.

TABLE I

Pertinent data on subjects of present study

Subject	Sex	Age	Diet			Urinary uric acid	Serum uric acid
			Calories	Protein	Purine N		
		yrs.	*C.*	*gm.*	*mg.*	*mg./d.*	*mg. %*
J. W.	M	27	2,200	50	4	476 ± 7*	5.4 ± 0.1*
S. N.	F	52	2,065	45	4	400 ± 13	4.3 ± 0.1

* Values represent mean ± probable error of the mean.

line of the hemilogarithmic plot of isotope abundance *vs.* time were calculated, together with their probable errors, by the method of least squares. The probable error of the miscible pool was calculated as previously described (10).

RESULTS

Effect of phenylbutazone on miscible pool, turnover rate, serum concentrations and urinary excretion of uric acid

The results of the study on J. W. are presented in Figure 1 and Tables II and III. During the control period a miscible pool of 1272 mg. of uric acid was obtained with a turnover rate of 0.456 pool, or 580 mg. of uric acid per day. This was 104 mg. greater than the urinary excretion of urate which averaged 476 mg. per day. When phenylbutazone was administered, there was a marked uricosuric response, beginning the second day and reaching a maximum of 837 mg. on the fifth day. Concurrently, the serum urate concentration fell from a mean of 5.4 mg. per cent to a low of 1.5 mg. per cent on the eighth day of drug

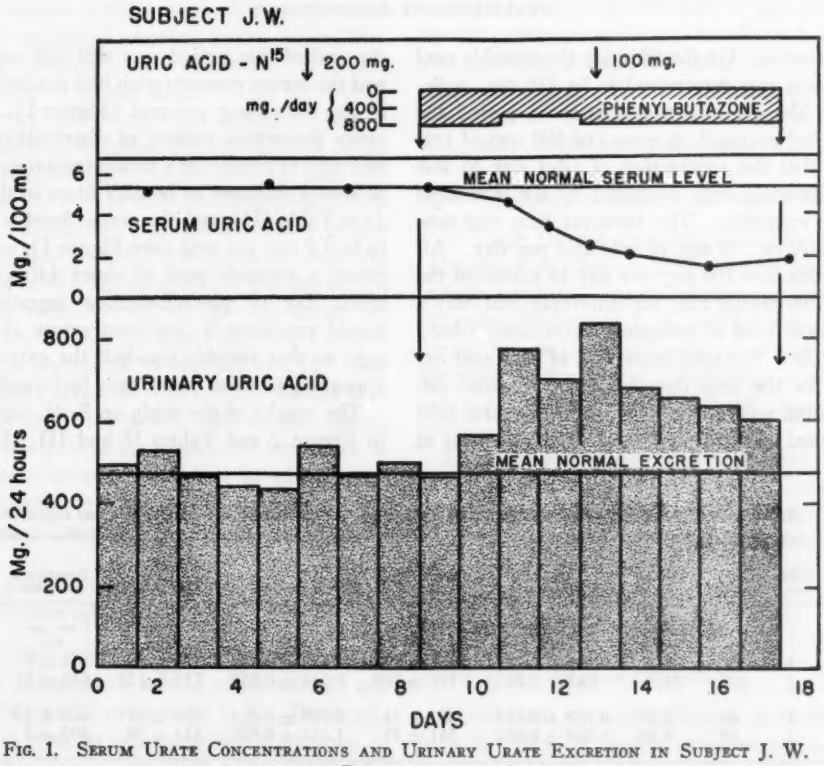

FIG. 1. SERUM URATE CONCENTRATIONS AND URINARY URATE EXCRETION IN SUBJECT J. W. BEFORE AND DURING PHENYLBUTAZONE ADMINISTRATION

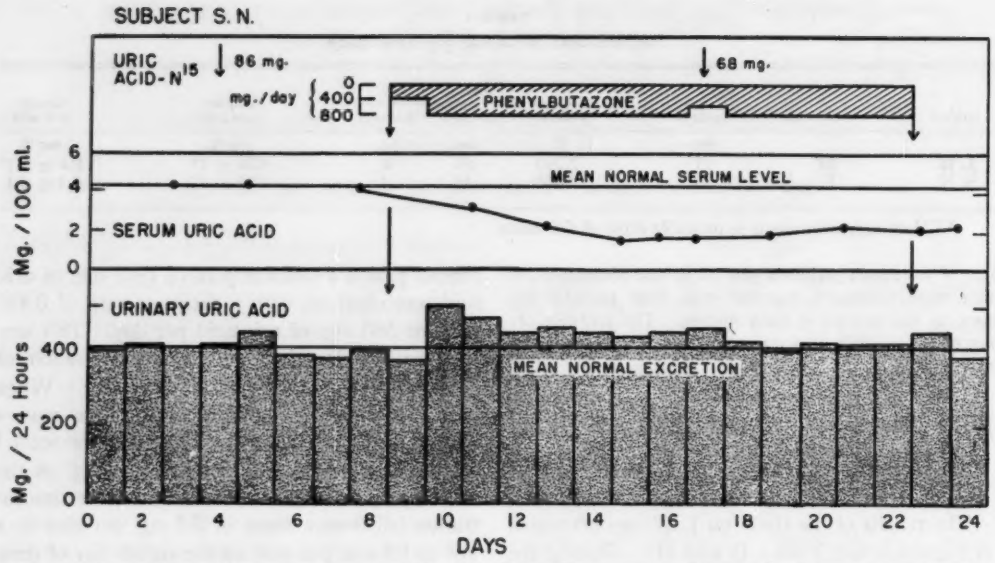

FIG. 2. SERUM URATE CONCENTRATIONS AND URINARY URATE EXCRETION IN SUBJECT S. N. BEFORE AND DURING PHENYLBUTAZONE ADMINISTRATION

administration. On the fifth day the miscible pool of uric acid was determined to be 716 mg., a decline of 556 mg. During this same period the subject had excreted an excess of 598 mg. of uric acid so that the contracture of pool size to this point was adequately explained by the increment in urate excretion. The turnover rate was now 1.084 pools or 776 mg. of uric acid per day. Although this was 196 mg. per day in excess of the control production rate, the difference had only a 69 per cent level of statistical significance (dev./p.e. = 1.5). The total increment of uric acid excretion for the nine days of phenylbutazone administration was 1630 mg., a quantity greater than the original miscible pool itself. Urinary urate at the end of this period was still 600 mg. per day and the serum concentration had reached a plateau at less than 2 mg. per cent (Figure 1). If an average theoretical volume of distribution in which uric acid is present at a concentration equal to that of serum, is taken to be 25.9 liters in this subject (see Table III) and the serum level is estimated to be 1.7 mg. per cent (see Figure 1), one can calculate a miscible pool of about 440 mg. on the ninth day of phenylbutazone ingestion. This would represent a pool contracture of only 832 mg., so that roughly one-half the extra uric acid appearing in urine must have had another origin.

The results of the study on S. N. are presented in Figure 2 and Tables II and III. During the

TABLE II

Magnitude of miscible pool of uric acid and its turnover before and during phenylbutazone ingestion

Subject	Exper. no.	a = dose injected	I_1 = isotope injected	I_0 = antiln of intercept	A = miscible pool	K = -slope	KA = turnover	B = urinary excretion	KA − B = surplus
		mg.	*atom per cent excess*	*atom per cent excess*	*mg.*	*day*$^{-1}$	*mg. per day*	*mg. per day*	*mg. per day*
J. W.	1	200	28.3	3.85 ± 0.15*	1272 ± 58	0.456 ± 0.008	580 ± 28	476 ± 7	104 ± 28
	2	100	28.3	3.47 ± 0.46	716 ± 108	1.084 ± 0.050	776 ± 122	644 ± 11	132 ± 123
S. N.	1	86	6.50	0.758 ± 0.035	651 ± 34	0.769 ± 0.012	501 ± 27	400 ± 13	101 ± 30
	2	68	6.50	1.085 ± 0.087	341 ± 32	1.211 ± 0.020	413 ± 39	405 ± 5	8 ± 39

* Values represent the mean ± probable error of the mean.

TABLE III

Volume of distribution of uric acid before and during phenylbutazone ingestion

Subject	Experiment no.	Miscible pool	Serum level	Theoretical volume of distribution	Per cent of body water*
		mg.	*mg. %*	*liters*	%
J. W.	1	1272	5.4	23.6	50.8
	2	716	2.5	28.6	
S. N.	1	651	4.3	15.1	56.9
	2	341	1.7	20.0	

* Body water was assumed to be 62 and 52 per cent of body weight for the male and female subjects, respectively (17). Owing to fluid retention during phenylbutazone ingestion, these assumptions could not be applied in the second experiments.

control period a miscible pool of 651 mg. of uric acid was obtained with a turnover rate of 0.769 pool or 501 mg. of uric acid per day. This was 101 mg. greater than her urinary excretion of urate which averaged 400 mg. per day. When phenylbutazone was administered, there was only a meager uricosuric response, with a peak excretion of 511 mg. being recorded on the second day. The daily urinary urate excretion remained slightly above the mean control value for a total of eight days, and during this period the serum urate concentration declined from 4.3 to 1.7 mg. per cent. On the ninth day the miscible pool was found to be 341 mg. of uric acid, a decline of 310 mg. The subject had excreted an excess of 359 mg. during this period. The turnover rate was 1.211 pools or 413 mg. of uric acid per day at this time. This value was 88 mg. per day below the control value, but this difference had only a 78 per cent level of significance (dev./p.e. = 1.8). The urinary urate excretions recorded for the last several days of this study were clearly normal values, so that in this patient the cumulative increment in urinary urate excretion incident to phenylbutazone administration correlated nicely with the decline in size of the miscible urate pool.

Effect of phenylbutazone on extra-renal disposal and catabolism of uric acid

In the control uric acid-N^{15} studies, the total recoveries of isotopic uric acid in urine were 76 per cent in J. W. and 73 per cent in S. N. These values were in good agreement with the portions of the daily turnover of uric acid accounted for by urinary urate excretion in these same subjects, which were 82 and 80 per cent, respectively. The residual 18 to 27 per cent not accounted for by either of these methods were in good agreement with similar determinations in other normal subjects (16, 18) and represented those portions of the daily turnovers disposed of by extra-renal pathways or catabolized to ammonia and urea (10).

Of the uric acid-N^{15} injected during phenylbutazone administration, 93 per cent was recovered unchanged in urine in both subjects. These values are clearly considerably greater than those procured prior to drug administration and they exclude the occurrence of increased extra-renal disposal or catabolism of uric acid in these subjects while on phenylbutazone. A comparison of the rates of synthesis and of excretion of uric acid during phenylbutazone ingestion (Table II) confirms this conclusion. In subject J. W. there was no significant change in the urate fraction escaping renal excretion which was now 17 per cent of the amount synthesized, whereas in subject S. N. there was a decided drop in this value to 2 per cent of that synthesized. In neither instance, however, was there any evidence for increased extra-renal disposal or catabolism of uric acid.

Effect of phenylbutazone on the uric acid space

In Table III are recorded the volumes of distribution of miscible urate for the two experiments in both subjects. The calculated uric acid spaces increased 5.0 liters in J. W. and 4.9 liters in S. N. during the periods from onset of phenylbutazone ingestion to the second administration of labeled uric acid, which were five and nine days, respectively.

The reduction of serum urate concentrations in S. N. and the expansion of urate space may be attributed in part to sodium and fluid retention (19, 20). During phenylbutazone ingestion, S. N.'s weight increased from 51.4 to 53.0 kg., and her hematocrit declined from 41 to 37 per cent. No such observations were made on J. W.

Toxic effects of phenylbutazone

There were no toxic effects of phenylbutazone in S. N. In J. W. gastric irritation and a pruritic maculo-papular eruption of the hands and feet,

together with slight fever, occurred on the third day and persisted throughout the study.

DISCUSSION

In previous studies (16) of the miscible pool of uric acid and the rate of its turnover, it has been shown that good straight lines were obtained if the logarithm of the abundance of N^{15} in urinary uric acid were plotted against time, after intravenous injection of small amounts of uric acid-N^{15}. This finding has been interpreted as supporting the assumptions made in the derivation of the expression

$$Kt = \ln (I_o/I)$$

where I_o is the isotope concentration in body uric acid at the moment of mixing, I the isotope concentration at time, t, thereafter, and K the fraction of the miscible pool replaced by unlabeled uric acid per unit time. On a semilogarithmic plot, $K = -$ slope and $\ln I_o =$ intercept. The antilogarithm of the intercept is used to calculate the size of the miscible pool, A, by the conventional isotope dilution equation (21)

$$A = a(I_1/I_o - 1)$$

where a is the quantity of uric acid injected and I_1 its isotope concentration. The product KA is the quantity of unlabeled uric acid entering the miscible pool per unit time.

In the normal subject on a purine-free diet, it is assumed that dilution of isotope within the miscible pool of uric acid occurs solely as a consequence of the ingress of newly synthesized unlabeled urate molecules. In gouty subjects, however, this decline of isotope abundance also reflects the entrance into the miscible pool of unlabeled molecules derived from solid urate deposits (22) and for this reason, K, obtained from treatment of isotope data in such subjects, no longer can be considered exclusively a rate constant of urate biosynthesis. For this reason, since the rate of synthesis of uric acid was a subject of investigation in the present study, gouty patients could not be employed, and observations were made on normal persons instead.

The results of the urate turnover studies in these two subjects give no support to the hypothesis (8) that phenylbutazone may suppress the rate of urate synthesis. Furthermore, such a postulate was unnecessary in these subjects since the contractures of the miscible pool due to the action of phenylbutazone were entirely attributable to augmented urinary excretion. Nor was any evidence procured favoring increased extra-renal excretion or enhanced catabolism of urate, for the recoveries of labeled uric acid in urine were actually greater during phenylbutazone ingestion than prior to it. Also, the percentage of the urate turnover appearing as urinary uric acid remained unchanged in one subject and was decidedly increased in the other (dev./p.e. = 1.96). It is probable that the rates of extra-renal disposal and catabolism of urate were reduced due to the diminution of serum urate concentration effected by phenylbutazone.

The theoretical uric acid space increased by about 5 liters in each subject during the interims between infusions of labeled urate. This was a 21 per cent expansion in J. W. and a 32 per cent expansion in S. N. In neither case was this change sufficient to explain more than a small portion of the suppression of serum urate concentration observed. The origin of this expansion of urate space is uncertain. In subject S. N. at least part of it would appear to be due to expansion of extracellular fluid volume, judging from the weight gain and drop of hematocrit. The percentage of body water initially occupied by the uric acid space, as calculated for these two subjects, is in very close agreement with the same values as recalculated from Benedict and co-workers (16, 22), which ranged from 46.8 to 55.1 per cent of total body water in four normal male subjects.

One further finding deserves comment. In subject J. W. a total of 1630 mg. of "extra" uric acid was excreted during nine days of phenylbutazone administration, a quantity larger than the original miscible pool which clearly cannot have been its sole source. Bishop, Rand, and Talbott (23) noted the same finding in two hyperuricemic subjects, one of them gouty, who were given Benemid®. In these subjects one might attribute the extra uric acid to mobilization of deposits not included within the miscible pool, but this seems an unlikely explanation in a normal subject. The author (24) has not observed continued excretion of "extra" uric acid in other normouricemic subjects given Benemid® or phenylbutazone. A more

probable explanation for the observation made on J. W. is that the rate of urate biosynthesis increased during the later days of phenylbutazone ingestion, as a consequence of the increased rate of metabolism accompanying fever. Taken in their entirety, the results procured in these subjects favor the view that phenylbutazone has only one important direct action upon uric acid metabolism, *viz.*, the enhancement of urinary urate excretion.

Yü, Sirota, and Gutman (6) have concluded that phenylbutazone is a uricosuric drug which depresses tubular reabsorption of urate. Uricosuria occurred in all of 10 gouty subjects given phenylbutazone intravenously, and the magnitude of this effect appeared to correlate with dosage. C urate/C inulin × 100 rose from a mean control of 6.04 to 19.5 within two hours; Tm_{PAH} was markedly suppressed in two subjects so studied. Seven of eight gouty subjects given phenylbutazone orally in dosages of 800 mg. per day showed increased urinary urate excretion. All exhibited a decrease in plasma urate concentration. In three an apparent inconsistency was observed between the degree of uricosuria and the decline in plasma urate concentration. In two of these there was a distinct fall in plasma urate concentration before the urinary urate excretion was appreciably increased; in one of these two, expansion of extracellular fluid volume consequent upon retention of salt and water was demonstrated. In the third subject, a small uricosuric effect lasted only one day, but the plasma urate concentration declined to 4.6 mg. per cent on the fourth day, the control value being 8.1 mg. per cent. Smyth has also found phenylbutazone a uricosuric drug in gouty subjects (4), as have Bishop and Beecher (7).

Kidd, Boyce, and Freyberg (5) did not find a significant increase in urinary urate excretion in the first 24 or 48 hours after the administration of 400 to 800 mg. of phenylbutazone to nine gouty subjects on uncontrolled diets, even though 12 of 15 patients showed a significant decrease in serum urate level. Johnson and co-workers (8) found a decrease in uric acid output in two gouty subjects on constant diets given 1 gm. of phenylbutazone daily, and failed to find increases in urinary urate in other gouty subjects in whom reductions of serum urate concentrations were observed. The analytic methods employed in both of these studies are not specific for uric acid, and possess inherent limitations which compromise the interpretation of results obtained with them (25, 26). Excretion of urinary chromogens affecting these methods may have been altered in such a manner as to obscure the actual uric acid changes.

It is apparent from Figure 2 that a highly specific and sensitive method for urate determination and rigorous consistency of purine intake are necessary to evaluate changes in urate excretion that may occur during phenylbutazone therapy. Seemingly minor changes may be adequate to explain the serum urate suppression, as this case demonstrates. Also, the sodium and fluid retention caused by phenylbutazone (19, 20) may lead to dilution of plasma urate and a considerable reduction in plasma urate concentration. It seems not improbable that a combination of non-specific analytical methods, inconstancy of dietary purine contribution to urinary urate excretion, and lack of recognition of extracellular fluid volume expansion may account for the observations which failed to demonstrate a correlation between decline of serum urate concentration and appearance of extra urinary urate.

SUMMARY

Uric acid-N^{15} has been administered to two normal subjects on purine-free diets during control periods and again during phenylbutazone ingestion. From a comparison of the magnitudes of contracture of the miscible pools and the cumulative increments in urinary urate excretions, it has been concluded that the uricosuric effect of phenylbutazone adequately explains the observed suppressions of serum urate concentrations. The rates of urate biosynthesis did not change significantly during the periods of study. Also, extra-renal disposal and catabolism of urates as measured by urinary recovery of N^{15} in uric acid were reduced in both subjects, or as measured by the percentage of the urate turnover appearing as urinary uric acid, were unchanged in one and reduced in the other subject. These changes were probably secondary to the marked reduction in serum urate concentration. The uric acid space increased by about 5 L. in both subjects, but this expansion explained only a small portion of the

suppression of serum urate concentration; a part of this increase was attributed in one subject to retention of fluid and expansion of extracellular fluid volume. Taken in their entirety, the results of this study indicate that phenylbutazone has only one important effect on uric acid metabolism in normal subjects, *viz.*, the enhancement of urinary urate excretion.

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VARIATIONS OF BETA GLUCURONIDASE CONCENTRATION IN ABNORMAL HUMAN SYNOVIAL FLUID[1]

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Enzyme studies of human synovial fluid have revealed the presence of amylase (1), a protease (2), and lipase (1), and a peroxidase (2). Alkaline phosphatase (3) has also been found and more recently, the enzyme aminotripeptidase (4) has been demonstrated. No studies have been made of the β glucuronidase content of human synovial fluid. In view of the significance of the enzyme in mucin metabolism (5) it was felt that an investigation of this problem would be of interest.

The present report represents a study of the β glucuronidase concentration of synovial fluids obtained from patients with various pathologic joint diseases. In several instances, serial assays were made in different patients suffering with degenerative joint disease, rheumatoid arthritis, or pyogenic arthritis. It will be shown that significant variations of enzyme concentration are found in these different types of joint disease.

METHOD

Synovial fluid was obtained from human patients by aspiration of the knee, or elbow joint. The aspirated fluid was placed in a bottle containing sufficient potassium oxalate to delay clot formation. As soon as possible 1.0 millilitre aliquots of this material were pipetted into each of two tests tubes. In one tube, serving as the control, 4.0 millilitres of 0.1 M acetate buffer of pH 4.5 was added. Three and five-tenths millilitres of 0.1 M acetate buffer was added to the second tube. The test tubes were then placed in a 37° C. waterbath. After a sufficient time had elapsed for temperature equilibration of the solution of joint fluid, 0.5 millilitres of 0.005 M phenolphthalein glucuronide[3] was added to the second tube. A few drops of chloroform were added to each tube to inhibit bacterial growth. Both tubes were incubated in the 37° C. waterbath for 16 to 24 hours. At the conclusion of the incubation period, 5.0 millilitres of 0.1 M glycine buffer of pH 10.5 was pipetted into each tube. Both were rapidly centrifuged to sediment particulate material. The supernatant fluid was then removed and assayed for phenolphthalein concentration. A Lumetron colorimeter with a 540 filter was employed, with the colorimeter set for the corresponding blank of each fluid to be analyzed. By interpolation from a previously prepared standard curve, the concentration of phenolphthalein was then calculated. The concentration of β glucuronidase was then expressed as one microgram of phenolphthalein liberated per millilitre per hour equal to one unit. This method of glucuronidase assay is a modification of that described by Talalay, Fishman, and Huggins (6).

RESULTS

Fifty-six patients with various pathologic joint disorders have been studied. In some instances serial determinations have been made on the same patient over a period of several months. The experimental results obtained from all assays of joint fluid are shown in Figure 1. It will be observed that patients with severe rheumatoid arthritis had considerable β glucuronidase in the synovial fluid while those that had a mild and slowly progressive rheumatoid arthritis had less β glucuronidase. In contrast, the data obtained from patients with degenerative joint disease show that with a single exception, these synovial fluids had significantly less β glucuronidase than any of the patients with rheumatoid arthritis.

Three patients with pyogenic arthritis were studied. It will be noted that the enzyme concentration in these individuals was greatly elevated, rising to values a thousand times those seen in degenerative joint disease. In patients with other types of joint disease (Figure 1), it will be observed that one individual with gout No. 5, one with psoriasis and arthritis No. 1, and one with a Charcot joint No. 6 had values somewhat higher than observed in patients with degenerative joint disease. Two patients with a traumatic joint ef-

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[3] Obtained from the Sigma Chemical Co., 4648 Easton Avenue, St. Louis 13, Missouri.

fusion, T, one with uremia No. 2 and one with an unexplained joint effusion No. 4 had low concentrations of β glucuronidase. One patient with a proliferative synovitis No. 3 also had a low concentration of synovial fluid β glucuronidase.

Data on three patients on whom serial determinations of glucuronidase were made are shown in Figure 2. The first patient was a 72-year-old man admitted to the Municipal Hospital with an acute febrile illness characterized by mild delirium, an acute monoarticular arthritis, and cutaneous lesions suggestive of meningococcal bacteremia. Cerebrospinal fluid examination was negative and several blood cultures showed no growth of bacteria. The left elbow was swollen, red and painful and there was marked restriction of joint motion. An aspiration of the joint revealed purulent fluid, which contained 123,000 W. B. C. per $mm.^3$, most of which were polymorphonuclear leucocytes. This fluid showed no bacterial growth after culture aerobically, anaerobically, and under CO_2. After therapy for several days with penicillin, a gradual fall of fever and a slow subsidence of joint swelling and improvement of joint motion occurred. However, the patient developed a severe, exfoliative dermatitis due to penicillin sensitivity. During this reaction, the left knee became swollen, red and tender. An aspiration of fluid from this joint revealed a cell count of 5,600 W. B. C. per $mm.^3$ After ACTH therapy, both the dermatitis and arthritis improved. On cessation of ACTH therapy, a recrudescence of joint swelling involving both knees, and wrists, and left elbow occurred.

While the etiology of the arthritis in this patient is not unequivocally defined, it was felt that the patient had an acute bacterial infection, possibly due to a meningococcus, which caused a suppurative arthritis of the left elbow. Subsequently this disease developed into multi-articular involvement which was more characteristic of a rheumatoid arthritis.

β glucuronidase studies of the elbow joint fluid aspirations (indicated by the solid circle, Figure 2) revealed very high levels of enzyme activity. After chemotherapy, a decrease in enzyme concentration was found. With later recrudescence of joint swelling, an increase of β glucuronidase was observed.

In the second patient, the data for β glucuronidase determinations are illustrated successively in a 68-year-old woman with an acute, severe rheumatoid arthritis. Initially this patient had little relief with systemic cortisone therapy or by local instillation of Hydrocortone® into both knee joints. Three months before her first clinic visit, and shortly after she sustained a hip fracture, the patient developed multi-articular pain, swelling and increased limitation of joint function. During the next six months there was progressive, severe acute joint disease involving both knees and the left wrist.

The first observation of the β glucuronidase concentration of the knee fluid (indicated by open circles, Figure 2) was made in July, 1953. At this time 50 mgm. of cortisone, administered by mouth, afforded moderate relief of joint symptoms. However, the patient required occasional supplementary injections of compound F into the left knee during August. In September, 1953, the patient developed more pain and effusion of the

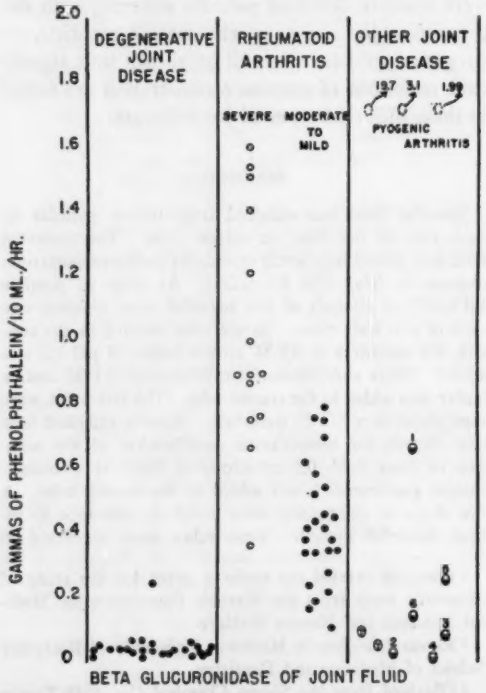

FIG. 1. SYNOVIAL FLUID CONCENTRATION OF β GLUCURONIDASE FOUND IN VARIOUS TYPES OF JOINT DISEASE

wrist and knees and other joints became painful and swollen. After increasing the cortisone dosage to 75 mgm., some improvement resulted, but her symptoms again increased in November. In November, Butazolidin® therapy was instituted at a time when cortisone was being gradually reduced. This therapeutic change produced more joint pain and disability. In early December, 50 mgm. of cortisone each day was re-instituted and Butazolidin® discontinued. By the end of December, while still taking 50 mgm. of cortisone each day, the patient began to improve. She was less depressed and all the previously swollen and tender joints, excepting the right knee, were less painful and swollen. By January, 1954, she was better than she had been in months.

The β glucuronidase determinations made periodically throughout this six-month interval reflected the severity of the arthritic process. During September (65th day of observation, Figure 2), when the peak of clinical activity was greatest, the highest concentration of enzyme was found. This was followed by a decrease in β glucuronidase to a value of 0.42 units during January, 1954 (180th day—Figure 2) when clinical improvement was evident.

The third patient is a 64-year-old woman with severe bilateral, degenerative joint disease involving both knees. She had considerable symptomatic relief of pain with monthly injections of Hydrocortone® into the synovial space of each knee. It will be noted that the β glucuronidase concentration in the synovial fluid of this patient was considerably less than in the patient with rheumatoid arthritis. The enzyme remained consistently low during the period of observation (lower curve, Figure 2).

These data suggest that the concentration of β glucuronidase reflects the severity of inflammation within the synovium and joint space. In patients with septic arthritis the enzyme is greatly increased. In patients with degenerative joint disease or traumatic effusion, low concentrations are found. The rheumatoid arthritic patients were divided into mild and severe forms of disease by evaluation of the duration and degree of symptoms of active inflammation, degree of progressive joint destruction and by determination of the erythro-

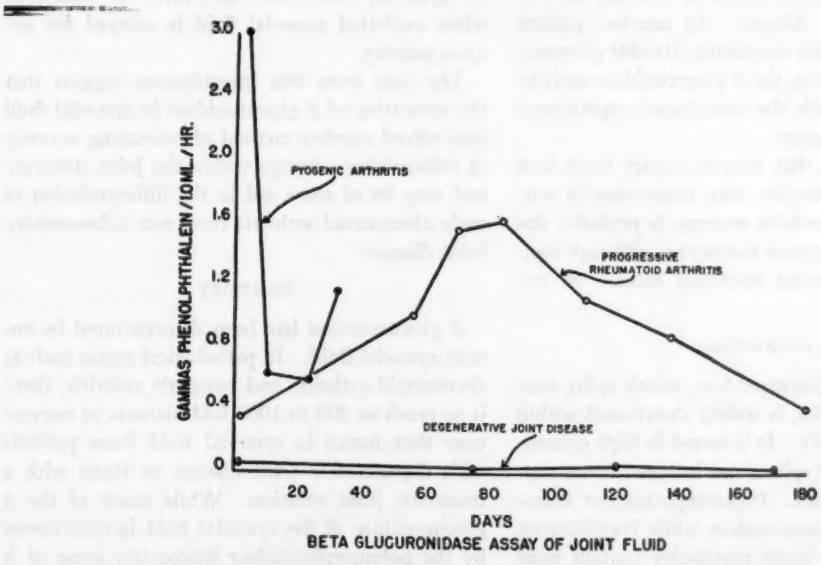

FIG. 2. RESULTS OF SUCCESSIVE DETERMINATIONS OF β GLUCURONIDASE CONCENTRATION IN THE SYNOVIAL FLUIDS OF PATIENTS WITH A) PYOGENIC ARTHRITIS (SOLID CIRCLE), B) RHEUMATOID ARTHRITIS (OPEN CIRCLE), AND C) DEGENERATIVE JOINT DISEASE (SEMI-CLOSED CIRCLE)

cyte sedimentation rate, plasma alpha globulin and fibrinogen concentrations, degree of anemia and extent of radiographic bone and joint alteration. With such a clinical estimation it was observed that higher concentrations of synovial fluid β glucuronidase are usually found in the patient with severe rheumatoid arthritis than in the mild, subacute disease (Figure 1).

A comparison of β glucuronidase concentration with the leucocyte count of joint fluid revealed some correlation. If the synovial fluid leucocyte count was very elevated, high concentrations of enzyme were found. If a few leucocytes were present, only small amounts of β glucuronidase were found. However, no absolute linear relationship was observed.

Joint fluid obtained from patients with severe rheumatoid arthritis and with septic arthritis was centrifuged to remove nearly all the formed elements. The supernatant fluid was then analyzed for β glucuronidase activity. In one instance, the untreated joint fluid had a β glucuronidase activity of 3.09 units. The centrifuged supernatant fluid had an activity of 0.76 units or nearly ten times the concentration found in synovial fluid of degenerative joint disease. In another patient with a septic arthritis containing 216,000 polymorphonuclear leucocytes, the β glucuronidase activity was 19.7 units, while the centrifuged supernatant fluid assayed 6.15 units.

It thus appears that enzyme, apart from that contributed by leucocytes, may accumulate in synovial fluid. This soluble enzyme is probably derived from disintegrated leucocytes, although contributions by synovial secretion cannot be excluded.

DISCUSSION

The enzyme β glucuronidase, which splits conjugated glucuronides, is widely distributed within the human body (5). It is found in high concentration in liver and spleen and in lower concentrations in other tissues. Polymorphonuclear leucocytes contain β glucuronidase while lymphocytes, platelets, and red blood corpuscles contain none (5). Pleural and ascitic effusions due to various pathologic disorders have been shown to contain β glucuronidase (5). This enzyme is also found in tears, and in gastric and bronchial secretions (5).

The present investigation reveals that synovial fluid obtained from human patients with various joint disorders also contains β glucuronidase. Individuals with degenerative joint disease and those with traumatic effusions or unexplained effusions had enzyme concentrations ranging between 0.015 to 0.080 units. Only one exception outside of this range was observed. Patients with rheumatoid arthritis had values ranging from 0.190 to 1.0 units. In general, those patients with the most severe rheumatoid arthritis had the highest values within this range, whereas the mild rheumatoid patients had concentrations of β glucuronidase in the lower part of this range. Patients with septic arthritis had concentrations of β glucuronidase as high as 19.7 units.

The concentration of β glucuronidase in synovial fluid appears to reflect the cellular response to the inflammatory change in the synovial membrane. Not only is the enzyme contained within the leucocytes, but it is also found in the cell-free fluid. Thus the contribution of intact leucocytes, disintegrated leucocytes and possible contribution by synovial membrane structures is measured when unclotted synovial fluid is assayed for enzyme activity.

The data from this investigation suggest that the estimation of β glucuronidase in synovial fluid may afford another method of estimating severity of inflammatory change within the joint structure and may be of some aid in the differentiation of early rheumatoid arthritis from non-inflammatory joint disease.

SUMMARY

β glucuronidase has been demonstrated in human synovial fluid. In pathological states such as rheumatoid arthritis and pyogenic arthritis, there is as much as 200 to 1000-fold increase of enzyme over that found in synovial fluid from patients with degenerative joint disease or those with a traumatic joint effusion. While much of the β glucuronidase of the synovial fluid is contributed by the polymorphonuclear leucocytes, some of it is soluble. The soluble fraction may be derived from disintegrated leucocytes or from secretion by the synovial membrane.

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THE RENAL RESPONSE TO CHRONIC RESPIRATORY ACIDOSIS[1]

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An increase in the carbon dioxide tension of the body fluids is accompanied by an increase in the concentration of bicarbonate bound base, a fact which minimizes the change in pH that would otherwise ensue (1, 2).

A priori, it is evident that an elevated plasma bicarbonate concentration can be maintained only by enhancement of the rate of renal tubular reabsorption of bicarbonate. That such enhancement occurs in response to acute respiratory acidosis has been established by experiments performed in our laboratory as well as in other laboratories (3–5). It has also been shown that the elevated pCO_2, and not the change in pH, is the stimulus responsible for enhancing this tubular activity. However, the data reported in these experiments on dogs indicate that the magnitude of the enhancement of tubular reabsorption of bicarbonate produced by acute respiratory acidosis is not sufficient to account for the elevated plasma concentrations frequently observed in states of chronic carbon dioxide retention in man. This suggests the possibility that some variety of tubular adaptation occurs in chronic respiratory acidosis which requires time for its development and which therefore is not seen in acute conditions.

METHODS

A total of 30 experiments was performed on eight mongrel female dogs. For each dog data on bicarbonate reabsorption at normal pCO_2 and during acute respiratory acidosis were obtained prior to the start of chronic experiments. The animals were maintained in metal screened metabolism cages covered by plastic hoods. Compressed air and CO_2 flowed into the cages at rates sufficient to insure adequate oxygenation. The relative flow rates of the two gasses were adjusted so as to maintain a CO_2 concentration of 10 to 14 per cent within the cages. This CO_2 concentration was determined periodically using a Cambridge hot-wire analyzer. Cages were opened for 5 to 10 minutes each day for feeding and cleaning.

Prior to the clearance periods, the dog was removed from the cage and anesthetized with sodium pentobarbital in doses not exceeding 30 mg. per Kg. An endotracheal airway was inserted and connected, *via* a Digby-Leigh non-rebreathing valve, to a Douglas bag containing 12 per cent CO_2 and 88 per cent O_2 which was breathed throughout the clearance periods. In most of the experiments the dog breathed spontaneously throughout. In a few experiments spontaneous respirations were abolished with a curarizing agent and ventilation was maintained by a high capacity respiration pump.[3] Details of the analytical methods and calculations have been given in a previous publication from this laboratory (3).

RESULTS

The effect of chronic respiratory acidosis on the renal tubular reabsorption of bicarbonate bound base was determined in twenty-four experiments. Duration of acidosis in these experiments varied from 12 hours to 77 days. Data from two such experiments are presented in Table I. In these experiments, creatinine and mannitol in an approximately isotonic solution were infused during the first two periods. In the subsequent periods, plasma bicarbonate was progressively elevated by suitable priming doses and sustaining infusions of sodium bicarbonate.

Experiment No. C-1 in Table I illustrates the effect of fifteen days of chronic respiratory acidosis on the renal reabsorption of bicarbonate.

During the first two periods, prior to the infusions of bicarbonate, the plasma bicarbonate concentration was supranormal. As illustrated in the last two columns of this table, renal reabsorption of bicarbonate was also elevated significantly. Reabsorption was essentially complete; excretion was virtually nil. With the infusion of sodium bicarbonate, plasma bicarbonate concentration rose

[1] Supported by a grant from the Life Insurance Medical Research Fund.

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TABLE I

Experiments illustrating the effect of chronic respiratory acidosis on the renal tubular reabsorption of bicarbonate bound base

Concurrent time *min.*	Glom. filt. rate *ml./min.*	Urine flow *ml./min.*	Plasma pH	Plasma $BHCO_3$ *mEq./L.*	Plasma pCO_2 *mm. Hg*	Urine pH	Urine $BHCO_3$ *mEq./L.*	Urine pCO_2 *mm. Hg*	$BHCO_3$ Filt. *mEq./min.*	$BHCO_3$ Excr. *mEq./min.*	$BHCO_3$ Reabsorbed *mEq./min.*	$BHCO_3$ Reabsorbed *mEq./100 ml. glomerular filtrate*
Experiment C-1. Dog C—17 Kg. 15 days chronic respiratory acidosis. Respiring 12 per cent CO_2–88 per cent O_2 during clearance periods												
−45 −38			Infuse: creatinine 1.4 gm./L.; mannitol 50 gm./L. @ 10.0 ml./min. Prime: creatinine 1.4 gm.									
0–15	90.5	6.16	7.21	35.5	91.7	6.62	8.68	84.9	3.38	0.053	3.33	3.68
15–35	90.5	6.13	7.22	35.6	89.7	6.27	4.07	89.4	3.38	0.025	3.36	3.70
35			Infuse: creatinine 1.4 gm./L.; $NaHCO_3$ 5.0 gm./L.; mannitol 25.0 gm./L. @ 10.0 ml./min. Prime: $NaHCO_3$ 5.0 gm.									
65–80	113.0	8.06	7.29	44.6	95.6	7.32	39.0	76.0	5.29	0.31	4.98	4.41
80–95	110.0	7.50	7.31	44.6	91.4	7.24	30.4	71.2	5.16	0.23	4.93	4.48
95			Infuse: creatinine 1.4 gm./L.; $NaHCO_3$ 10.0 gm./L. @ 10.0 ml./min. Prime: $NaHCO_3$ 5.0 gm.									
120–135	112.0	9.80	7.39	54.0	92.0	7.59	71.4	74.9	6.35	0.70	5.65	5.04
135–150	115.0	8.66	7.42	54.3	86.4	7.63	73.5	70.3	6.36	0.64	5.92	5.15
150			Infuse: creatinine 1.4 gm./L.; $NaHCO_3$ 20.0 gm./L. @ 10 ml./min. Prime: $NaHCO_3$ 5.0 gm.									
185–200	96.5	9.73	7.49	64.8	87.7	7.73	123.8	93.9	6.56	1.20	5.36	5.55
200–215	99.4	9.66	7.51	65.3	84.4	7.74	125.0	93.9	6.81	1.21	5.60	5.64
Experiment C-7. Dog N—16.5 Kg. 46 hours chronic respiratory acidosis												
−75 −70			Infuse: creatinine 1.0 gm./L.; mannitol 50.0 gm./L. @ 10.0 ml./min. Prime: creatinine 1.3 gm.									
0–15	40.5	5.17	7.21	31.3	80.8	6.15	2.38	68.9	1.33	0.01	1.32	3.26
15–30	38.0	5.77	7.19	31.8	86.0	6.13	2.52	75.0	1.27	0.01	1.26	3.32
30			Infuse: creatinine 1.0 gm./L.; $NaHCO_3$ 6.0 gm./L.; mannitol 25.0 gm./L. @ 10.0 ml./min. Prime: $NaHCO_3$ 4.0 gm.									
60–75	40.7	6.27	7.26	37.5	86.4	6.67	7.19	62.4	1.60	0.04	1.56	3.82
75–90	40.9	6.63	7.28	37.2	81.4	6.66	6.71	59.8	1.60	0.04	1.56	3.80
90			Infuse: creatinine 1.0 gm./L.; $NaHCO_3$ 12.0 gm./L. @ 10.0 ml./min. Prime: $NaHCO_3$ 4.5 gm.									
120–135	42.0	5.80	7.40	49.1	81.7	7.53	45.3	54.4	2.16	0.26	1.90	4.53
135–150	43.5	5.71	7.41	51.9	84.4	7.60	58.9	60.2	2.37	0.34	2.03	4.67
150			Infuse: creatinine 1.0 gm./L.; $NaHCO_3$ 18.0 gm./L. @ 10.0 ml./min. Prime: $NaHCO_3$ 4.5 gm.									
180–195	47.5	7.60	7.46	60.8	88.4	7.74	114.9	84.2	3.04	0.87	2.17	4.57
195–210	47.7	7.93	7.47	64.2	91.0	7.75	130.3	93.8	3.21	1.03	2.18	4.57

progressively to 65 mEq. per L. in the last period. Renal tubular reabsorption of bicarbonate also rose to reach a value in the last period of 5.64 mEq. per 100 ml. filtrate. In contrast, the average value for the normal dog is 2.5 mEq. per 100 ml. filtrate, and for the dog in acute respiratory acidosis, with a comparable elevation of carbon dioxide tension, it is 3.3 mEq. per 100 ml. filtrate (3, 6).

Essentially similar results were obtained after 46 hours of respiratory acidosis, as is shown in experiment No. C-7 in Table I. Here, prior to the infusion of bicarbonate, the renal reabsorption of bicarbonate averaged 3.29 mEq. per 100 ml. fil-

trate in the first two periods. With progressive increase in plasma bicarbonate, renal bicarbonate reabsorption rose and became essentially constant during the last four periods, the average value being 4.58 mEq. per 100 ml. filtrate.

In Figure 1 are presented the data on renal bicarbonate reabsorption, as a function of plasma bicarbonate concentration, obtained in this group of experiments. In this graph the mean values for bicarbonate reabsorption under conditions of normal pCO_2 and in acute respiratory acidosis are indicated by the appropriately labeled horizontal lines. It is apparent that in the range of plasma bicarbonate concentration at which frank excretion occurs; *i.e.*, above 40 mEq. per liter, the renal reabsorption of bicarbonate in chronic respiratory acidosis is significantly greater than in either control or acute experiments. Inspection of the data of individual experiments reveals considerable splay in the "titration" curve; *i.e.*, saturation of the mechanism of bicarbonate reabsorption is approached gradually over a wide range of plasma bicarbonate concentration. This is in contrast to observations made when plasma carbon dioxide tension is normal or acutely increased. Under these conditions, a similar "titration" of the tubules by progressive elevation of plasma bicarbonate concentration yields a curve with a relatively sharp angle at that plasma concentration at which saturation of the mechanism occurs.

Data on the renal reabsorption of bicarbonate bound base, as a function of the duration of respiratory acidosis, are presented in Figures 2A and 2B. In Figure 2A are plotted the rates of reabsorption of bicarbonate bound base obtained prior to the infusion of sodium bicarbonate, and in Figure 2B, the maximal rates of reabsorption attained during bicarbonate loading. Although there is considerable scatter in the data, it is apparent that the increase in renal reabsorption in respiratory acidosis occurs relatively rapidly in the first 24 to 48 hours; *i.e.*, from a mean acute value of 3.3 to approximately 4.6 mEq. per hundred ml. of filtrate, with a relatively slower increase thereafter. It is also apparent that the capacity of the renal tubular mechanism to reab-

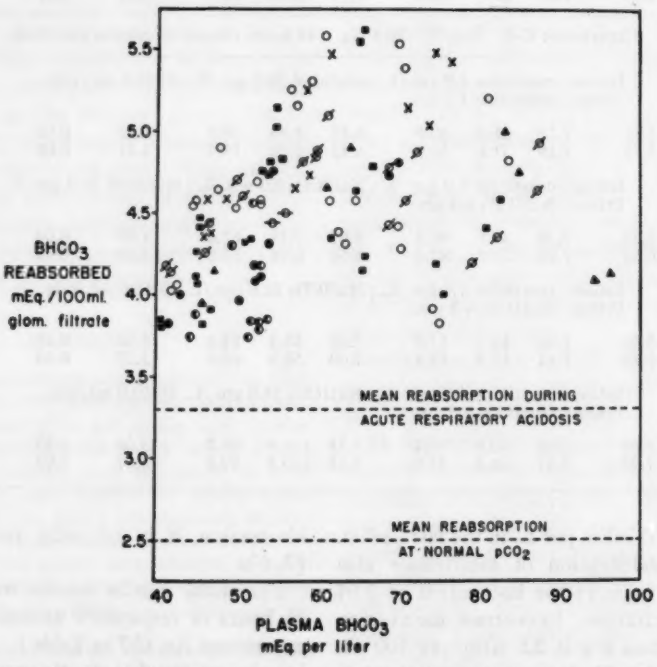

FIG. 1. THE RENAL TUBULAR REABSORPTION OF BICARBONATE AS A FUNCTION OF PLASMA BICARBONATE CONCENTRATION

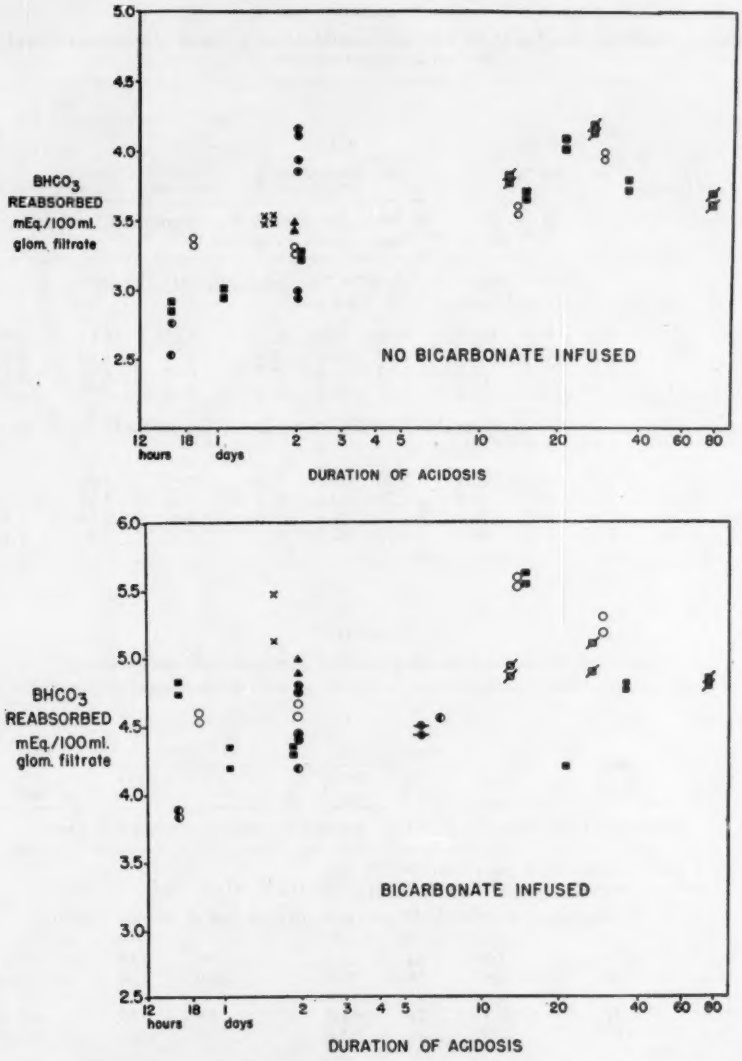

FIG. 2. RENAL TUBULAR REABSORPTION OF BICARBONATE AS A FUNCTION OF DURATION OF ACIDOSIS

A—PRIOR TO THE INFUSION OF BICARBONATE,
B—DURING BICARBONATE LOADING

sorb bicarbonate, as indicated by the rates attainable during bicarbonate loading (Figure 2B), is significantly greater than would be indicated by the plasma bicarbonate concentration attained during respiratory acidosis, before any bicarbonate is infused (Figure 2A).

Inhibitors of the enzyme carbonic anhydrase, such as the sulfonamide compound No. 6063, employed clinically under the proprietary name Diamox®,[4] have been shown to depress renal tubular reabsorption of bicarbonate. This depression has

[4] Lederle.

TABLE II

Experiment illustrating the effect of No. 6063 on renal tubular reabsorption of bicarbonate bound base in chronic respiratory acidosis

Concurrent time min.	Glom. filt. rate ml./min.	Urine flow ml./min.	Plasma pH	Plasma $BHCO_3$ mEq./L.	Plasma pCO_2 mm. Hg	Urine pH	Urine $BHCO_3$ mEq./L.	Urine pCO_2 mm. Hg	$BHCO_3$ Filt. mEq./min.	$BHCO_3$ Excr. mEq./min.	$BHCO_3$ Reabsorbed mEq./min.	$BHCO_3$ Reabsorbed mEq./100 ml. glomerular filtrate
Experiment C-17. Dog M—21.5 Kg. 48 hours chronic acidosis. Respiring 12 per cent CO_2–88 per cent O_2 during clearance periods												
−135			Infuse: creatinine 1.2 gm./L.; $NaHCO_3$ 15.0 gm./L. @ 10.0 ml./min.									
−125			Prime: creatinine 1.8 gm.; $NaHCO_3$ 8.0 gm.									
0–15	89.6	12.4	7.28	52.0	114.0	7.66	92.6	82.5	4.89	1.15	3.74	4.17
15–30	86.0	14.8	7.47	52.1	73.8	7.69	94.4	78.4	4.70	1.40	3.30	3.84
30–45	83.5	15.2	7.49	52.2	70.5	7.75	94.0	68.0	4.58	1.43	3.15	3.78
45–60	78.4	14.4	7.50	53.2	70.5	7.76	94.4	67.0	4.38	1.36	3.02	3.85
60			Infuse: creatinine 1.0 gm./L.; $NaHCO_3$ 18.0 gm./L.; No. 6063 0.72 gm./L. @ 10.0 ml./min. Prime: No. 6063 0.22 gm.									
85–95	61.2	18.1	7.48	56.1	77.8	7.70	99.4	81.0	3.61	1.80	1.81	2.96
95–105	58.0	17.2	7.47	56.8	80.5	7.71	112.6	90.6	3.46	2.07	1.29	2.22
105–115	55.5	16.3	7.46	56.5	82.1	7.69	109.1	90.6	3.29	1.78	1.51	2.72
115–125	52.2	16.0	7.45	57.2	84.8	7.70	107.5	87.5	3.14	1.72	1.42	2.72

TABLE III

Bicarbonate reabsorption following cessation of chronic respiratory acidosis

Days post-acidosis	Glom. filt. rate ml./min.	Urine flow ml./min.	Plasma pH	Plasma pCO_2 mm. Hg	Plasma HCO_3 mEq./L.	Urine pH	Urine HCO_3 mEq./L.	$BHCO_3$ Reabsorbed mEq./min.	$BHCO_3$ Reabsorbed mEq./100 ml. glomerular filtrate
Experiment C-27. Dog S—15.0 Kg. Chronic respiratory acidosis—77 days									
			Prime: creatinine 0.8 gm.; $NaHCO_3$ 7.0 gm. Infuse: creatinine 0.9 gm./L.; $NaHCO_3$ 10 gm./L. @ 10 ml./min. Started approximately 45 min. prior to each pair of clearance periods						
0.25	54.4	6.27	7.57	63	55.9	7.96	140	2.31	4.25
	44.9	5.46	7.60	56	55.5	7.89	138	1.87	4.16
1	60.5	6.57	7.57	73	64.2	7.89	153	3.08	5.10
	58.6	5.80	7.59	66	62.8	7.91	167	2.89	4.93
3	49.3	4.33	7.72	40	51.6	7.91	145	1.96	3.98
	52.8	4.53	7.74	40	50.2	8.01	163	1.96	3.72
4	52.0	7.22	7.64	56	58.5	7.88	130	2.16	4.16
	37.4	5.75	7.68	50	57.9	7.90	143	1.39	3.72
7	45.7	6.00	7.67	50	55.4	7.87	128	1.71	3.74
	46.1	7.00	7.70	43	53.5	8.06	114	1.72	3.73
11	36.8	3.22	7.65	50	52.5	7.96	155	1.53	4.16
	34.8	2.90	7.67	46	52.1	7.95	164	1.43	4.10
14	43.3	5.97	7.54	53	45.0	7.74	92	1.49	3.44
	40.6	7.47	7.58	50	44.9	7.80	91	1.23	3.03

been demonstrated for conditions of normal pCO_2 (6) and for acute respiratory acidosis (3).

The effect of No. 6063 [5] on the renal reabsorption of bicarbonate in chronic respiratory acidosis is illustrated by the data presented in Table II. During the first four periods of this experiment, obtained following 48 hours of chronic respiratory acidosis, elevated plasma bicarbonate concentration with frank excretion was obtained by infusing sodium bicarbonate. During these periods, the usual enhancement of bicarbonate reabsorption associated with chronic respiratory acidosis was attained. Between periods four and five a priming dose of No. 6063 (10 mg. per Kg.) was given, followed by a sustaining infusion containing No. 6063 (20 mg. per Kg. per hr.). This resulted in a reduction in reabsorption of bicarbonate to values well below the control periods, but still increased with respect to values observed in the normal, non-acidotic state, or to values observed following No. 6063 in equal dosage during acute respiratory acidosis. Similar results were obtained in three other experiments. This suggests that the renal tubular adaptation which occurs in chronic respiratory acidosis is not specifically abolished by the enzyme inhibitor, rather that the drug exerts its usual action in blocking some fraction of enzymatically catalyzed reabsorption.

Studies were carried out in two dogs to determine the behavior of the renal mechanism for bicarbonate reabsorption during the period of recovery from chronic respiratory acidosis. Data obtained on one of these dogs in chronic respiratory acidosis for 77 days are presented in Table III. It will be noted that renal reabsorption of bicarbonate was elevated for 14 days following cessation of acidosis. Unfortunately, the dog died through an accident at this point. In another dog, in chronic respiratory acidosis for 21 days, bicarbonate reabsorption was still above normal on the sixth day, although in one period on the second day it approached normal.

Two factors complicate the interpretation of these results. First, the arterial pCO_2 in these experiments was somewhat elevated, generally falling in the range of 50 to 60 mm. Hg. This elevation occured despite minimal dosage of barbiturate, and may be a consequence of an altered sensitivity of the respiratory center to CO_2. However, from a comparison of these data with data previously obtained on bicarbonate reabsorption in this range of pCO_2 (3, 4), it appears unlikely that the enhanced reabsorption observed here is solely attributable to the elevation in pCO_2.

The second factor to be considered in interpreting these results is the moderate hypokalemia (1.7 to 3.5 mEq. per L.) usually associated with the enhancement in bicarbonate reabsorption. Since the dogs ate a normal diet and therefore presumably had a normal potassium intake, this implies either enhanced potassium excretion or an alteration in transcellular distribution of potassium. Whether the hypokalemia reflects a net depletion of body potassium stores, as is generally the case (7–9), and whether it is causally related to the enhanced bicarbonate reabsorption, cannot be determined from these data. In any event, the renal mechanism for bicarbonate reabsorption continues to function at a supranormal level for some time after the initiating stimulus—the inhalation of CO_2—has been removed.

DISCUSSION

The data presented above indicate that chronic respiratory acidosis results in an enhancement of renal tubular reabsorption of bicarbonate bound base to rates significantly greater than those attained in response to acute respiratory acidosis. This adaptation appears to proceed most rapidly during the first 24 to 48 hours of respiratory acidosis, and less rapidly thereafter. Some hormonal mechanism, possibly involving the adrenal cortex, may be involved. The acute administration of cortisone to dogs with normal plasma pCO_2 did not result in a significant enhancement of bicarbonate reabsorption (10). This does not, however, preclude the possibility that adrenal steroids, acting for prolonged periods in chronic respiratory acidosis, might act to enhance renal tubular reabsorption of bicarbonate. Preliminary results obtained by Giebisch (11) suggest that acute respiratory acidosis in dogs pretreated with moderately large doses of desoxycorticosterone acetate and cortisone, together with a high dietary salt intake, results in enhancement of bicarbonate

[5] We are indebted to the American Cyanamid Corporation for a supply of No. 6063.

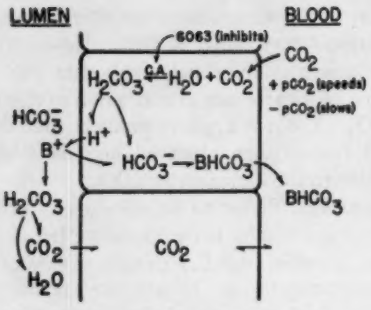

FIG. 3. PROPOSED SCHEMA FOR REABSORPTION OF BICARBONATE

reabsorption to values within the range of those observed by us in chronic respiratory acidosis.

The schema outlined in Figure 3 has recently been proposed (3, 4) to explain the renal tubular reabsorption of bicarbonate bound base by an ion exchange mechanism. The intracellular hydration of CO_2, enzymatically accelerated by carbonic anhydrase, yields carbonic acid. The dissociation of this acid produces hydrogen ions which are then exchanged across the cell membrane for ions of base bound by bicarbonate ions in the tubular urine. The carbonic acid thus formed in the tubular lumen dehydrates in part yielding molecular CO_2 which may then diffuse back across the cell membrane and re-enter the reabsorptive cycle, as pointed out by Brazeau and Gilman (4). The ions of base are returned to the peritubular blood accompanied by bicarbonate ions formed through the dissociation of carbonic acid within the cell.

Since the renal reabsorption of bicarbonate in acute respiratory acidosis has been found to be a function of the pCO_2 of the body fluids, it has been proposed that an elevation in the pCO_2 within the tubular cells, by a mass action effect, accelerates the hydration reaction and consequently enhances bicarbonate reabsorption.

From an inspection of the data it is apparent that, for a given pCO_2, bicarbonate reabsorption in chronic respiratory acidosis is significantly greater than it is in acute respiratory acidosis. In the range of arterial pCO_2 of 70 to 110 mm. Hg, the mean of many determinations of bicarbonate reabsorption in chronic respiratory acidosis was 4.14 mEq. per 100 ml. filtrate; whereas the mean of determinations in acute respiratory acidosis, similarly distributed over the same range, was 3.43 mEq. per 100 ml. filtrate.

We have noted above that in chronic respiratory acidosis there is increase in the splay of the curve relating reabsorption of bicarbonate to plasma bicarbonate concentration. This may indicate an alteration in the kinetics of the ion-exchange mechanism such that the reaction whose velocity determines the over-all rate of hydrogen ion transport is different from the rate-limiting reaction in conditions of normal or acutely elevated CO_2 tension (12, 13). One alternative explanation would be that there is a greater dispersion of glomerular activity in chronic respiratory acidosis.

Table IV summarizes data relating bicarbonate reabsorption to plasma potassium concentration in a variety of experimental conditions. It is apparent that prior to bicarbonate infusion, no correlation exists between bicarbonate reabsorption and plasma potassium concentration. Furthermore, no correlation is evident during infusion of bicarbonate, although plasma potassium always decreased as a result of such infusion. Obviously differences in bicarbonate reabsorption between animals in acute and chronic respiratory acidosis cannot be related to changes in plasma potassium concentration.

Berliner, Kennedy, and Orloff (14) have postulated that potassium and hydrogen ions compete in the renal tubular cell for some common secretory mechanism. One might therefore anticipate that

TABLE IV

Renal bicarbonate reabsorption and plasma potassium concentration in various experimental conditions

		Mean $BHCO_3$ reabsorption *mEq./100 ml. filtrate*	Plasma potassium Mean ±2 X S.D. *mEq./L.*
No bicarbonate infused	Normal pCO_2	2.24	3.76 ± 0.88
	Acute respiratory acidosis	2.90	3.75 ± 0.04
	Chronic respiratory acidosis	3.47	3.62 ± 0.74
Bicarbonate infused	Normal pCO_2	2.58	2.78 ± 0.90
	Acute respiratory acidosis	3.47	2.90 ± 0.42
	Chronic respiratory acidosis	4.61	2.68 ± 0.88

the intracellular concentration of potassium rather than the plasma concentration would be the factor determining bicarbonate reabsorption, if indeed potassium is to be accorded any significance. If cellular potassium were low, the concomitant increase in rate of secretion of hydrogen ions would account for a part of the enhanced reabsorption of bicarbonate bound base. On the other hand, a normal or increased cellular potassium concentration would lend support to the concept that the adaptation to chronic respiratory acidosis involves an enhancement of the mechanism for secreting hydrogen ions.

We have no data on potassium concentration in renal tubular cells. Muscle potassium in rats in chronic respiratory acidosis is in the high normal range (15). One might argue that, if renal tubular cells in the dog are similar, they are not depleted of potassium. However, no unequivocal statement is possible until data on potassium in renal tubular cells in chronic respiratory acidosis are available.

From a consideration of the reactions diagrammed in Figure 3, it may be concluded that there are at least two processes in the reabsorptive mechanism, acceleration of which could result in an enhanced bicarbonate reabsorption. The first of these processes is the hydration of CO_2 within the cell to produce carbonic acid. For a given pCO_2, acceleration of this reaction would require an increase in the activity of renal carbonic anhydrase. The other process, the kinetics of which might be affected by this adaptation, consists of those energy-requiring reactions involved in the transport of hydrogen ions across the cell membrane. The nature of this process is not known at present.

From our data, it is not possible to specify which of the reaction velocities has been altered during the adaptation to chronic respiratory acidosis, or whether a change in more than one is involved. Calculations based on available data concerning the carbonic anhydrase activity present in normal kidneys (16) are subject to a large degree of uncertainty. However, these calculations suggest that the amount of carbonic anhydrase normally present suffices to account for the most rapid rates of bicarbonate reabsorption observed in our studies. It is therefore suggested that the adaptation to chronic respiratory acidosis probably involves the mechanism for the transport of hydrogen ions.

The available data concerning possible changes in renal enzyme activities in response to respiratory acidosis are conflicting. Killion and Schaefer (17) exposed rats and guinea pigs to atmospheres containing 30 per cent CO_2, and reported significant increases in renal carbonic anhydrase activity occurring within one hour. However, Seldin (18) in preliminary studies on rats in an atmosphere containing 10 per cent CO_2 for periods of two weeks, was unable to demonstrate any significant increase in renal carbonic anhydrase activity. A more precise description of the nature of the renal enzymatic adaptation to chronic respiratory acidosis awaits further study.

SUMMARY AND CONCLUSIONS

1. The renal reabsorption of bicarbonate bound base in chronic respiratory acidosis is enhanced to values significantly greater than those observed either under conditions of normal pCO_2 or in acute respiratory acidosis.

2. The enhancement of bicarbonate reabsorption varies as a direct function of the duration of acidosis, rising most rapidly during the first 24 to 48 hours. This may occur as the result of a hormonal adaptation, possibly involving the adrenal cortex.

3. These results have been discussed in terms of a schema originally proposed on the basis of results obtained in acute respiratory acidosis. Since, for a given pCO_2, bicarbonate reabsorption in chronic respiratory acidosis is significantly greater than in acute respiratory acidosis, some adaptation involving the kinetics of the reactions concerned is implied. It is suggested that this probably involves the mechanism for the secretion of hydrogen ions.

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THE EFFECT OF A SINGLE ORAL DOSE OF THE CARBONIC ANHYDRASE INHIBITOR, ACETAZOLEAMIDE, IN RENAL DISEASE

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The carbonic anhydrase inhibitor acetazoleamide, (2-acetylamino-1,3,4-thiadazole-5 sulfonamide, 6063, Diamox®), is believed to decrease the exchange of hydrogen for sodium ions in the renal tubule (1). Alkalinization of the urine with sodium, potassium, and bicarbonate diuresis is found after its administration, both in normal people and in patients with cardiac failure (2). Except for the report by Metcoff, James, and Gordillo in nephrosis (3), no data are available concerning the effect of acetazoleamide in primary renal disease. This investigation was undertaken to see if the effect in patients with renal disease was the same as in normal people, and to evaluate any possible therapeutic benefit from the use of the drug.

The action of acetazoleamide is believed to be limited to the carbonic anhydrase enzyme system, and this suggested that it could be used to determine the presence of functioning renal carbonic anhydrase. The occurrence of the customary urine changes after administration of acetazoleamide would then indicate the presence of functioning carbonic anhydrase in the kidney.

MATERIAL AND METHODS

The subjects used for this investigation included four healthy young males and fifteen patients who were selected because of the presence of severe renal disease. Each patient's clinical status was steady at the time of study. None had diarrhea or vomiting. The individual diagnoses are shown in Table I, with clinical data and details of tests obtained within a few days of acetazoleamide administration, in the Appendix. All subjects were in bed overnight and were in a basal post-absorptive state, remaining recumbent throughout the procedure. An indwelling urethral catheter containing multiple holes in the tip was inserted into the bladder at least two and one-half hours before the start of the first collection period in all subjects, except in the case of the normal males M. K. and J. B. who voided spontaneously under oil. Exposure of the urine to air was avoided in all. In the first eleven cases studied, the catheter was connected by a short length of rubber tubing and the urine allowed to drain into a container under a layer of mineral oil. In the last six cases, urine was withdrawn directly into oiled 50 cc. syringes. Specimens were kept on ice until analysed the same morning. Water, 120 cc. to 300 cc. depending on body weight, was taken orally at 5 a.m. and thereafter hourly until 1 p.m. The first collection period started at 8:30 a.m., each lasted one hour, the bladder being emptied by firm suprapubic pressure at the end. After two control periods, approximately 10 mg. per kg. of acetazoleamide[2] were given orally, and three more collection periods obtained, the last one ending at 1:30 p.m. The entire procedure was tolerated without discomfort. Blood for non-protein nitrogen and bicarbonate was taken during the control period.

The range of urine specific gravity was determined from multiple random analyses and in addition either a Mosenthal, or a concentration and dilution test. The excretion of phenolsulfonphthalein following intravenous injection was determined in the usual manner. Urine was collected by catheterization.

The chemical methods employed were for serum nonprotein nitrogen, Evelyn's modification of Folin-Wu; sodium and potassium by internal standard flame photometry; bicarbonate in plasma and urine by the volumetric method of Van Slyke and Cullen (4) after equilibrating with CO_2 at a tension of 40 mm. Hg. Although this technique is not ideal, the difference between the bicarbonate estimated at a pCO_2 of 40 mm. Hg and that actually existing is small, and does not influence these results. Blood pH was not determined, but it is believed that under the conditions in which these patients were studied, a low serum bicarbonate indicated the presence of metabolic acidosis. Urine pH was determined at room temperature using a Beckman meter and a 290–31 anaerobic electrode. Chloride was determined by the technique of Wilson and Ball (5); phosphorus by that of Fiske and Subbarow (6). Titratable acidity was determined by the method of Henderson and Palmer (7), the end point being checked using a pH meter. Ammonia nitrogen was determined by the continuous aeration technique (8).

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[2] Supplied through the courtesy of Dr. J. D. Gallagher, Lederle Laboratories Division, American Cyanamid Company.

TABLE I

Showing the effect of acetazoleamide on the urine

Patient	Diagnosis	Group	Serum HCO_3 *mEq./L.*	Vol. *cc./min.* C*	Vol. *cc./min.* A†	*μEq./min.* pH C	pH A	Na C	Na A	K C	K A	Cl C	Cl A	HCO_3 C	HCO_3 A	PO_4 C	PO_4 A	NH_4 C	NH_4 A	H C	H A
J. R.	Normal	I	27.2	1.25	2.91	6.11	7.6	20.7	201.4	7.3	110.0	27.4	127.5	2.95	129.2	4.5	42.0	128	76	60	0
C. V.	Normal		27.0	4.77	5.22	7.08	7.66	141.2	402.0	73.1	197.5	151.7	108	62.9	443.0	6.8	41.5	194.5	119.3	81	0
J. B.	Normal		27.4	5.54	8.26	6.59	7.45	181.0	608.0	53.2	138.0	170.0	270.2	30.2	399.0	41.9	58.4	130.0	99.1	190	0
M. K.	Normal		28.4	5.15	6.70	6.81	7.53	208.1	508.2	85.9	132.2	256.6	215.4	35.2	359.3	2.8	20.7	209	95.6	87	0
Mean change from control level‡				1.59 ±0.46		0.91 ±0.20		292.1 ± 51.3		89.5 ± 16.5		28.8 ± 41.1		299.8 ± 59.1		26.6 ± 5.4		−68.1 ± 17.8		−104 ± 29.0	
J. S.	Malignant hypertension	II	27.4	1.71	3.33	6.29	7.20	89.8	249.9	37.5	74.1	101.6	158.6	4.8	110.2	23.3	38.8	175	126.5	109	51
F. S.	Chronic pyelonephritis		28.0	3.35	5.45	7.07	7.48	13.8	80.1	80.4	142.2	12.6	29.4	47.6	174.4	21.4	23.6	41.5	57.1	97	10
F. St.	Diabetic nephropathy		27.0	0.83	2.07	5.25	7.28	0.22	29.5	30.7	83.7	21.5	40.3	0.75	64.0	1.37	2.86	62.5	27.3	50	0
L. D.	Acute nephritis		27.4	3.83	4.14	6.84	7.5	113	132	78.5	157.5	143	102	16.4	141	22.6	34	75.2	29.5	180	16
R. G.	Acute nephritis		27.6	2.21	3.38	5.44	7.20	90.6	188.5	72.3	96.0	142.5	155.5	1.30	84.7	16.1	20.1	168	41.6	201	21
T. W.	Nephrotic syndrome		19.4	0.58	2.23	5.85	7.40	2.57	14.4	14.8	143.6	1.0	8.4	0.59	67.8	17.1	23.5	159	46.4	340	30
Mean change from control level				1.34 ±0.24		1.22 ±0.26		64.0 ± 23.3		63.7 ± 15.2		12.0 ± 12.8		95.1 ± 11.4		6.8 ± 2.2		−58.8 ± 21.4		−107 ± 27.0	
R. Y.	Diabetic nephropathy	III	19.0	1.34	1.54	6.49	7.02	43.9	49.1	35.3	46.9	41.0	40.0	6.6	16.3	17.6	18.7	13.5	8.2	76	39
J. B.	Chronic pyelonephritis		19.0	0.89	0.93	6.38	7.08	60.1	61.1	25.7	30.8	56.0	47.7	1.4	10.3	16.1	15.2	25.4	16.1	54	10
M. A.	Chronic pyelonephritis		18.7	2.73	3.56	6.40	6.60	83.2	83.4	56.2	42.4	83.5	73.6	4.4	8.1	22.5	23.7	141.5	102	191	173
F. L.	Chronic nephritis		15.2	1.64	1.43	5.35	6.0	160.5	132.0	30.3	28.7	147	126	1.4	1.8	23.9	21.0	46.8	28.1	222	137
A. S.	Carcinoma of cervix		12.3	0.61	0.79	5.11	5.55	3.5	7.5	48.2	50.4	5.5	14.3	0.3	1.1	20.7	27.5	138	96.3	230	210
P. H.	Chronic pyelonephritis		12.7	1.55	1.67	6.44	6.49	86.4	78.4	35.5	33.4	89.9	73.7	4.3	5.0	17.8	17.8	0	0	76	75
Mean change from control level				0.19 ±0.14		0.42 ±0.10		−4.3 ± 5.1		0.2 ± 3.4		−7.9 ± 4.3		4.0 ± 1.73		0.8 ± 1.3		−19.0 ± 7.2		−34 ± 11.9	
C. B.	Renal tubular acidosis	IV	20.1	1.33	2.12	6.54	7.45	14.7	58.8	21.3	34.1	21.7	22.7	4.7	57.3	16.7	13.3	49.8	22.7	70	0
M. B.	Renal tubular acidosis		14.0	3.1	2.27	7.01	7.1	71.9	71.0	36.9	49.7	84.6	100.5	16.8	10.7	27.5	28.5	210	221	12	52.2
	+ Sodium citrate		28.9	1.24	2.27	7.7	7.86	130.0	280.0	17.9	23.2	44.2	58.0	71.0	201.0	13.9	17.0	26.6	29.5	0	0
F. P.	Renal tubular acidosis		14.8	2.4	2.4	7.08	6.99	80.1	82.0	21.1	23.3	90.2	76.2	22	19.4	12.7	17.5	103	103	30	40
	+ Sodium citrate		24.7	3.33	3.21	7.42	7.46	207.5	267.0	18.7	55.3	128.5	88.2	83.5	210.5	14.8	24.1	89.6	57.3	10	0
Mean change from control level§				0.56 ±0.35		0.37 ±0.27		84.5 ± 33.0		18.2 ± 10.0		−8.5 ± 16.3		103.2 ± 25.3		3.0 ± 3.6		−18.8 ± 10.9		−26 ± 21.8	
J. B.	Normal + 374 mM NH_4Cl	V	16.1	5.04	4.27	4.75	5.0	140.5	108.8	106.5	134.5	341.5	300.5	1.53	1.50	61.5	53.6	1261	818	495	417
M. K.	Normal + 374 mM NH_4Cl		15.3	6.5	2.6	5.13	6.67	303.0	84.8	161.2	177.8	486.5	198.4	4.70	17.4	82.1	38.1	785	181	408	113
	Normal + {336 mM NH_4Cl 111 mM K Cl		16.9	4.39	4.18	4.82	6.78	117.2	60.5	129.5	185.5	305.5	182.5	2.4	22.8	26.9	45.3	643	191	301	222
Mean change from control level				−1.62 ±1.14		1.25 ±0.51		−102.2 ± 58.4		33.5 ± 11.7		−150.7 ± 72.6		10.9 ± 6.0		−11.1 ±18.0		−499 ± 52.2		−150 ± 72.1	
Probability of difference between means arising by chance																					
Group I *vs.* II				.7 >		.4 >		.01 >		.3 >		.8 >		.01 >		.02 >		.8 >		.9 <	
Group I *vs.* III				.05 >		.1 >		.001 >		.001 >		.5 >		.01 >		.01 >		.05 >		.1 >	
Group II *vs.* III				.01 >		.02 >		.02 >		.01 >		.2 >		.001 >		.05 >		.2 >		.05 >	

* C refers to the average rate of excretion per minute during the two control hours.
† A refers to the average rate of excretion per minute during the second and third hours after administration of acetazoleamide.
‡ The mean change above or below the control level ± the standard error is given for each group.
§ With M. B. and F. P. values found after sodium citrate were used in calculation of the mean change.

RESULTS

For comparison of the findings, the rates of excretion per minute found during the two control hours have been averaged and compared to the average values found during the second and third hours after giving acetazoleamide. The data are shown in Table I and presented graphically in Figure 1.

Group I—Normal subjects

The response to the administration of acetazoleamide in the four normal subjects was similar to that described previously in both man (2) and the dog (9), consisting of a moderate increase in urine volume and a rise in urine pH over 7.4 with diminution in titratable acid and ammonia. There was a marked increase in sodium and bicarbonate excretion with a smaller rise in potassium loss. Any change in chloride loss was inconstant but phosphate excretion increased slightly.

Group II—Renal disease without acidosis

There were six patients in this group all of whom had normal plasma bicarbonate levels except for T. W., in whom it was 19.4 mEq. per L. This was associated with vomiting before admission and the presence of a large empyema. All patients showed a response to the drug. There was, however, a significant decrease in sodium and bicarbonate loss as compared with Group I, with potassium tending to be the major cation accompanying the increase in excreted bicarbonate. This was most marked in the patients with the smallest increments in sodium excretion. Changes in urine volume, pH, titratable acid and ammonia were similar in the two groups.

Group III—Renal disease with acidosis

The six patients in this group all had metabolic acidosis caused by renal insufficiency, with plasma bicarbonates 19 mEq. per L. or less. The urinary

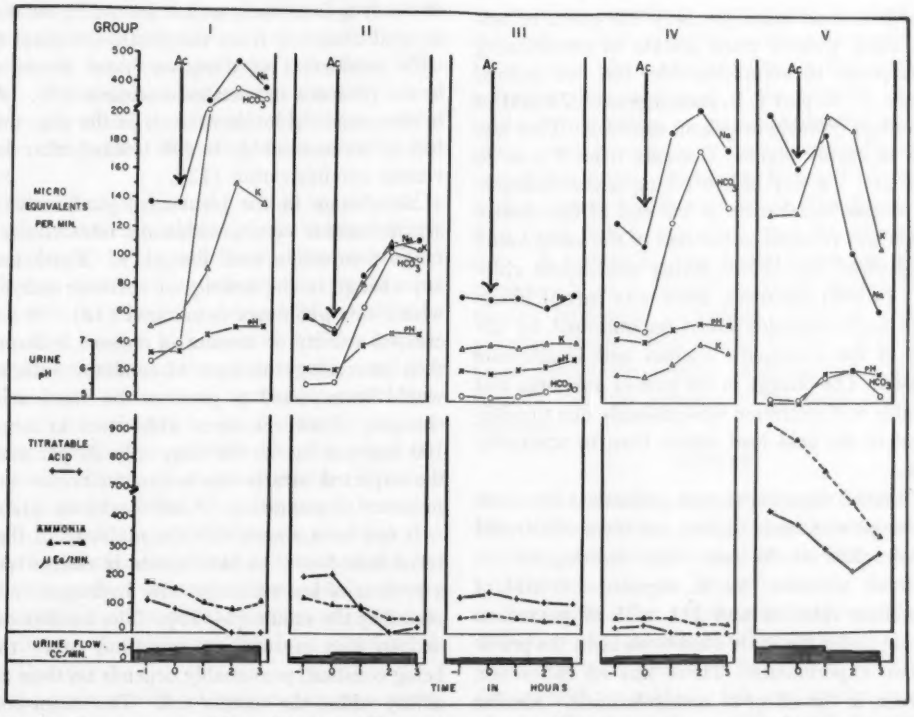

FIG. 1. SHOWING THE MEAN RATES OF EXCRETION IN EACH GROUP BEFORE AND AFTER ACETAZOLEAMIDE

changes after acetazoleamide were minimal or absent completely.

Group IV—Renal tubular acidosis

Three patients with this syndrome were studied. C. B. had a plasma bicarbonate level of 20.1 mEq. per L. and he showed the usual urinary changes after acetazoleamide. M. B. and F. P. with plasma bicarbonates of 14.0 mEq. per L. and 14.8 mEq. per L., respectively, showed no urinary changes after the inhibitor when acidotic, but definite changes after correction of the acidosis with sodium citrate. Comparison of the individual values of this group with the other groups is difficult owing to the effect of treatment superimposed on the basic urinary abnormality. Nevertheless, the increment in sodium and bicarbonate excretion after acetazoleamide is the same in magnitude in these three patients as in the patients in Group II.

Group V—Ammonium chloride acidosis

To determine more precisely the effect of acidosis alone without renal disease in conditioning the response to acetazoleamide, the two normal subjects M. K. and J. B. each ingested 374 mM of ammonium chloride in gelatin capsules. This was taken in evenly divided amounts from 9 a.m. to 10.30 p.m. the day before taking acetazoleamide. The plasma bicarbonate at the end of the control periods was reduced to one-half of the value found the previous day before taking ammonium chloride. In both instances, there was partial blocking of acetazoleamide effect as indicated by absence of the customary sodium and bicarbonate diuresis. The change in the rate of ammonia and titratable acid excretion was probably due to withdrawal of the acid load rather than to acetazoleamide.

A further experiment was performed in which an attempt was made to keep the intracellular pH normal whilst at the same time creating an extracellular acidosis. M. K. ingested 336 mM of ammonium chloride and 111 mM of potassium chloride under the same conditions as in the previous two experiments. There was no difference, however, in the effect of acetazoleamide. Similar depression of serum bicarbonate was produced in both instances and with the supplementary potassium, the serum potassium rose from 3.93 mEq. per L. to 6.51 mEq. per L.

DISCUSSION

The demonstration that metabolic acidosis blocks the effect of oral acetazoleamide in man is in complete agreement with the findings of Maren and Wadsworth in dogs (10). Reports of other workers stating that acetazoleamide exerts its customary action in metabolic acidosis (1, 11) are probably explained by the mild degree of acidosis in these experiments. From the present data, the blocking effect of metabolic acidosis was demonstrated in patients with renal disease when the plasma bicarbonate was 19 mEq. per L., or below. In the normal controls, this effect was observed when the plasma bicarbonate was in the neighbourhood of 16 mEq. per L.

Several possible mechanisms may be considered to explain the apparent lack of effect of acetazoleamide in metabolic acidosis. Impaired absorption of the drug is unlikely as it is known in the dog to be well absorbed from the gastro-intestinal tract with production of adequate blood levels even in the presence of metabolic acidosis (9). Also, in ammonium chloride acidosis in the dog, the effect of acetazoleamide is still blocked after intravenous administration (12).

No change in the pharmacological activity of acetazoleamide occurs within the intracellular pH range compatible with life (12). Furthermore, any change in the activity of carbonic anhydrase within this pH range is minimal (13). If an increased activity or amount of enzyme is assumed, then increasing the dose of inhibitor sufficiently would be expected to produce the usual urinary changes. However, even with doses as large as 100 mg. per kg. in the dog, only 20 per cent of the expected bicarbonate excretion occurs in the presence of ammonium chloride acidosis (12).

It has been shown that the majority of the filtered base bound to bicarbonate is reabsorbed by a process of ion exchange with hydrogen ions secreted by the tubule (14, 15). The number of hydrogen ions secreted at any time, other things being constant, presumably depends on their availability within the tubular cell. The major contribution to this supply will be from the hydration of

carbon dioxide, catalyzed by carbonic anhydrase as shown by the reaction

$$CO_2 + H_2O \overset{C.A.}{\rightleftharpoons} H_2CO_3$$
$$H_2CO_3 \rightleftharpoons H^+ + HCO_3^-.$$

If the concentration of hydrogen ions within the cell was increased secondary to changes in the extracellular fluid pH, then it would be expected that proportionally less of the secreted hydrogen ions would originate through the action of carbonic anhydrase. Inhibition of the enzyme with acetazoleamide might then have less effect on base reabsorption than usual.

Conversely, decreasing the intracellular hydrogen ion concentration by infusion of sodium bicarbonate would make the enzymatic hydration process the principal source of hydrogen ions. Inhibition of the enzyme under these circumstances indicates that a larger than usual amount of bicarbonate escapes reabsorption (16, 17).

Leaf, Schwartz, and Relman (18) have suggested that with reduction in the filtered load of bicarbonate, fewer hydrogen ions are required for adequate base reabsorption. Less response might therefore be seen after carbonic anhydrase inhibition. This does not explain the absence of acetazoleamide effect in ammonium chloride acidosis where high rates of hydrogen ion secretion are found.

With reduction in renal mass by disease, a decrease in the carbonic anhydrase content of the kidney would be expected. Enzyme inhibition might then cause smaller urinary changes as compared with the changes produced in normal controls. The smaller increment in sodium and bicarbonate excretion in the patients with renal disease without acidosis is compatible with this. However, the changes in urine volume, pH, potassium, ammonia, and titratable acid were not statistically different from the controls. This may be due to the small size of each group. It is also possible that although hydrogen ion secretion was effectively blocked, other tubular mechanisms ensured less sodium excretion than usual. In regard to this, the increased exchange of potassium for sodium is of interest and has been noted previously (19, 20).

In the patients with acidosis, the absence of any urinary changes after using acetazoleamide could be caused both by the acidosis itself and in addition by the other mechanisms discussed above.

The syndrome of idiopathic renal tubular acidosis has been suggested as being due to a deficiency of carbonic anhydrase in the renal tubules (1, 21). The excretion of an alkaline urine containing large amounts of base is similar to the urines produced after administration of acetazoleamide. In the three cases in this series, all showed evidence of functioning renal carbonic anhydrase. This suggests that some other metabolic abnormality, perhaps involving the transport of hydrogen ions rather than their production is at fault in this condition.

Except for transitory drowsiness and occasional headache, no side effects were observed in these patients. Even in those with advanced renal failure, no evidence of untoward effect was seen in the urine, blood pressure, or degree of azotemia, after the use of acetazoleamide. In view of the relative freedom from toxic manifestations to date, the drug might be considered as an adjunct in the treatment of patients with primary renal disease, or in other conditions complicated by renal disease where fluid retention exists. It might also be of value when alkalinization of the urine, or potassium loss is desired. The presence of azotemia, hypertension, or marked reduction in functional tests do not themselves contraindicate the use of the drug. However, if there is acidosis with a plasma bicarbonate below 19 mEq. per L. then there will be no effect from its administration. A further limiting factor in the use of acetazoleamide as a diuretic in renal disease is the smaller increment in sodium excretion as compared to that found in normal people. It does not seem possible to predict beforehand the magnitude of this loss because of the large individual variation.

SUMMARY

The effect of a single oral dose of the carbonic anhydrase inhibitor, acetazoleamide, was studied in fifteen patients with renal disease and four normal controls. In the normal subjects, there was an increase in urine volume, rise in urine pH, with sodium, potassium and bicarbonate diuresis. This effect was also produced in patients with renal disease without acidosis, but was smaller in magnitude. In the patients with renal disease and

metabolic acidosis however, the urinary changes were minimal or absent completely.

Following production of ammonium chloride acidosis in two normal subjects, acetazoleamide failed to produce the customary urinary changes.

The reason for these findings and the possible therapeutic value of acetazoleamide in renal disease was discussed.

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APPENDIX

Group II

J. S. 54–4008. Female, aged 39 years. Weight 70.0 kg. Hypertension discovered March 1954, blood pressure 240/140. Marked retinal changes with papilledema. Urinalysis showed protein +++, with red and white cells and casts. Culture was sterile. Retrograde pyelography was normal. Tested with 687 mg. acetazoleamide on April 17th, 1954. The non-protein nitrogen was 46 mg. per cent, phenolsulfonphthalein excretion (P.S.P.) 37 per cent at 60 minutes and range of urine specific gravity 1003 to 1021 at this time.

F. S. 53–19228. Male, aged 50 years. Weight 75 kg. Hypertension since 1940 at which time blood pressure 180/110, rising to 250/150 in 1953. Investigation in 1950 showed the urine to contain protein + intermittently, with a few white cells. P.S.P. excretion was 37 per cent in 30 minutes and the urine specific gravity was 1023 after a concentration test. The serum non-protein nitrogen was 27 mg. per cent, and creatinine 1.2 mg. per cent. A pyelogram was abnormal on the left side and bacterial growth was obtained from both ureteral urines on culture. He was readmitted in 1953 when the urinary concentrating ability was reduced to 1015, the 30-minute P.S.P. was 15 per cent, the serum non-protein nitrogen 29 to 52 mg. per cent and creatinine 1.91 mg. per cent. Rice diet was started on December 10th, 1953, and he was tested with 750 mg. acetazoleamide on December 23rd. The non-protein nitrogen was 38 mg. per cent, P.S.P. 23 per cent, at 60 minutes and range of urine specific gravity 1002 to 1015 at this time.

F. St. 54–5293. Female, aged 45 years. Weight 50 kg. Diabetes mellitus was discovered in 1947. Subsequent control was poor. In 1952, blood pressure 150/80, the urine contained frequent white cells. Urine culture was sterile and the specific gravity was fixed between 1004 to 1010. The non-protein nitrogen was 31 mg. per cent. In 1953, generalized edema developed with proteinuria ++. Her condition was worse in 1954, edema was still present, blood pressure 160/90. The serum proteins were 6.55 gm. per cent, albumen 3.03 gm. per cent, globulin 3.52 gm. per cent, cholesterol 448 mg. per cent. Tested with 500 mg. acetazoleamide on April 14th, 1954. The non-protein nitrogen was 55 mg. per cent, P.S.P. 10 per cent at 60 minutes, and range of urine specific gravity 1001 to 1013 at this time.

L. D. 53–18500. Male, aged 11 years. Weight 37 kg. Severe attack of acute nephritis in 1949 with apparent complete recovery. Readmitted on November 18th, 1953, with hematuria, proteinuria +++ and casts in the urine following a sore throat. Blood pressure was 110/64, non-protein nitrogen 80 mg. per cent, serum proteins 6.25 gm. per cent, albumen 2.5 gm. per cent and globulin 3.7 gm. per cent. Tested with 375 mg. acetazoleamide on November 25th, 1953. The non-protein nitrogen was 29 mg. per cent, and range of urine specific gravity 1003 to 1020 at this time. He was discharged clinically well on December 7th, 1954, but the urine still contained protein ++ and white cells.

R. G. 54–5394. Male, aged 21 years. Weight 77.2 kg. Admitted on April 2nd, 1954, with acute nephritis. The blood pressure was initially 172/135 and gradually fell to 130/85. Tested with 750 mg. acetazoleamide on April 28th, 1954. The non-protein nitrogen was 36 mg. per cent, P.S.P. 65 per cent at 60 minutes and range of urine specific gravity 1002 to 1014 at this time. He was discharged on May 17th, 1954, at which time the urine still contained a trace of protein and occasionally white cells.

T. W. 53–19849. Male, aged 4 years. Weight 20 kg. Onset of generalized edema in September, 1953, with proteinuria ++++, serum proteins of 4.51 gm. per cent, albumen 1.49 gm. per cent, globulin 3.02 gm. per cent, cholesterol 362 mg. per cent, and non-protein nitrogen 24 mg. per cent. Diuresis after receiving ACTH at this time. Readmitted on December 11th, 1953, with recurrence of edema, fever, and vomiting. Tested with 200 mg. acetazoleamide on December 16th. The non-protein nitrogen was 19 mg. per cent and range of urine specific gravity 1004 to 1030 at this time. Closed drainage of a large empyema was performed on December 22nd, and subsequently diuresis occurred.

Group III

R. Y. 53–4827. Female, aged 53 years. Weight 56.5 kg. Hypertension was discovered in 1945 and diabetes mellitus one year later. Blood pressure 196/108, proteinuria and diabetic retinopathy at this time. Onset of generalized edema in 1949, with proteinuria +++, and white cells and casts in the urine. The serum proteins were 4.78 gm. per cent, albumen 3.33 gm. per cent, globulin 1.45 gm. per cent, non-protein nitrogen 62 mg. per cent, and cholesterol 385 mg. per cent at this time. Her

condition was unchanged in 1954, but the non-protein nitrogen had risen to 107 mg. per cent. Tested with 562 mg. acetazoleamide on April 7th, 1954, when on 600 mg. sodium diet. The non-protein nitrogen was 110 mg. per cent, P.S.P. 5 per cent at 60 minutes and range of urine specific gravity 1005 to 1013 at this time. Developed a myocardial infarction and died on May 11th, 1954. Autopsy revealed advanced Kimmelstiel-Wilson lesions in the kidneys.

J. B. 54–3202. Male, aged 75 years. Weight 65 kg. Admitted in uremia, urine contained protein ++, and numerous red and white cells. Tested with 625 mg. acetazoleamide on March 10th, 1954. The non-protein nitrogen was 81 mg. per cent, P.S.P. 3.5 per cent at 60 minutes and range of urine specific gravity 1008 to 1012 at this time. Became jaundiced terminally and died on March 25th, 1954. Autopsy showed chronic pyelonephritis and post-necrotic hepatic cirrhosis.

M. A. 54–17891. Female, aged 14 years. Weight 37 kg. Neurogenic bladder with incontinence present since infancy. Urinalysis showed protein +, with frequent white cells. The hemoglobin was 50 per cent. Serum sodium 150.7 mEq. per L., potassium 5.29 mEq. per L., chloride 110 mEq. per L., bicarbonate 13 to 18 mEq. per L., calcium 3.4 mEq. per L., phosphorus 2.87 mEq. per L., and alkaline phosphatase 32.2 units. Urinary calcium was 0.7 to 2.3 mEq. per 24 hrs. on a 90 mEq. calcium intake. The urine pH fell to 6.1 only, after ammonium chloride, whilst the serum bicarbonate fell from 13.9 to 8.2 mEq. per L. Rachitic changes were present at all epiphyses radiologically. Tested with 375 mg. acetazoleamide on January 27th, 1954, before any treatment. The non-protein nitrogen was 78 mg. per cent and range of urine specific gravity 1003 to 1012 at this time.

F. L. 54–1270. Male, aged 53 years. Weight 74 kg. Urinalysis normal, blood pressure 104/58 and non-protein nitrogen 42 mg. per cent in 1945. Admitted in uremia in 1954. The urine contained protein +++, red and white cells and casts. Retrograde pyelography was normal. Tested with 750 mg. acetazoleamide on January 27th, 1954. The non-protein nitrogen was 111 mg. per cent, and range of urine specific gravity 1006 to 1016 at this time.

A. S. 54–456. Female, aged 46 years. Weight 50 kg. Carcinoma of the cervix was diagnosed in 1952. Blood pressure 138/84, urinalysis normal, non-protein nitrogen 22 mg. per cent and cystography and pyelography normal at this time. Received X-ray therapy. The carcinoma recurred in October, 1953, when complete obstruction of the left ureter with partial obstruction of the right ureter was demonstrated. The blood pressure was 160/100 and the non-protein nitrogen 22 mg. per cent. She was admitted with a subarachnoid hemorrhage and uremia on January 8th, 1954, and tested with 500 mg. acetazoleamide on January 20th, 1954. The non-protein nitrogen was 103 mg. per cent and range of urine specific gravity 1012 to 1017 at this time.

P. H. 54–1580. Female, aged 37 years. Weight 53 kg. Admitted January 27th, 1954, in uremia. The blood pressure was 150/80, urinalysis showed protein +++, with numerous red and white cells and casts. Tested with 500 mg. acetazoleamide on February 3rd, 1954. The non-protein nitrogen was 106 mg. per cent, P.S.P. 0 per cent at 60 minutes and range of urine specific gravity 1006 to 1012 at this time. Gradually deteriorated with rising blood pressure to 205/115 and died on April 27th, 1954. Autopsy showed chronic pyelonephritis, both kidneys weighing a total of 75 gm.

Group IV

C. B. C.M.H. 54–650. Male, aged 4 years. Weight 12.5 kg. Episodes of fever and vomiting since infancy. One of these in February, 1953, was associated with tetany. Admitted on February 6th, 1954, with pneumonia and carpopedal spasm. History of polydipsia, polyuria, and a high milk intake. Examination showed him to be small for his age. Urinalysis showed occasional white cells, pH 6.1 to 6.7. Urea clearance was 44 per cent of normal. Serum sodium 145 mEq. per L., potassium 5.2 mEq. per L., chloride 103.5 mEq. per L., carbon dioxide content 16.3 to 22.0 mEq. per L., calcium 2.9 mEq. per L. on admission and thereafter 4.9 mEq. per L., phosphorus 2.9 mEq. per L., and alkaline phosphatase 2 units. Radiological examination revealed bilateral nephrocalcinosis. There was no evidence of bone disease. Tested with 125 mg. acetazoleamide on March 1st, 1954. The non-protein nitrogen was 28 mg. per cent and range of urine specific gravity 1001 to 1007 at this time.

M. B. 54–5719. Male, aged 16 years. Weight 25.6 kg. Grew normally until aged 7 but had not grown since. Skeletal deformity was noted one year later. Examination showed him to be dwarfed with enlarged epiphyses. Blood pressure 118/50. Urinalysis negative. The urine pH was never below 7.0. Urea clearance was 28 per cent of normal. There was no amino aciduria. The serum proteins were 6.51 gm. per cent, sodium 141.5 mEq. per L., potassium 3.27 mEq. per L., chloride 114 mEq. per L., bicarbonate 12 to 15 mEq. per L., calcium 5.3 mEq. per L., phosphorus 1.36 mEq. per L., and alkaline phosphatase 106 units. There were marked rachitic changes at the epiphyses on radiological examination, and bilateral nephrocalcinosis was present. The excretion of titratable acid rose from 115 μEq. per min. to 391 μEq. per min. with a fall in urine pH from 7.0 to 6.7 after infusion of neutral sodium phosphate. The serum phosphate rose from 1.45 mEq. per L. to 6.2 mEq. per L., and the urinary phosphate excretion from 20 μEq. per min. to 167 μEq. per min. Tested with 250 mg. acetazoleamide before and then after taking sodium citrate. The non-protein nitrogen was 30 mg. per cent, P.S.P. 58 per cent at 60 minutes and range of urine specific gravity 1000 to 1012 at this time.

F. P. 54–2220. Female, aged 29 years. Weight 38 kg. She had passed urinary calculi since the age of 14. Always taken a high calcium diet. Blood pressure 110/65, urinalysis showed protein +, with a few white cells. The urine pH was never below 6.9. Urea clearance was 30 per cent of normal. The serum proteins were 7.85

gm. per cent, sodium 138 mEq. per L., potassium 3.13 mEq. per L., chloride 115 mEq. per L., bicarbonate 12 to 14 mEq. per L., calcium 4.9 mEq. per L., phosphorus 1.68 mEq. per L., and alkaline phosphatase 6.1 units. Radiological examination showed very advanced bilateral nephrocalcinosis but there was no evidence of bone disease. Tested with 375 mg. acetazoleamide before, and then after three months on sodium citrate. The non-protein nitrogen was 26 mg. per cent, P.S.P. 19 per cent at 60 minutes and range of urine specific gravity 1005 to 1010 at this time.

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URINARY ALPHA-KETOLIC STEROID METABOLITES OF CORTICAL HORMONES ADMINISTERED TO SUBJECTS WITH ADRENAL CORTICAL INSUFFICIENCY[1]

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The analysis of urinary excretory products following the administration of known compounds is one of the classical methods for the study of their metabolism. The developments in paper chromatographic (1) and micro-analytic (2) technics and the use of glucuronidase hydrolysis (3) have considerably decreased the difficulties of investigating steroid metabolism by this method. Using these newer technics, we have studied the urinary excretion pattern of the major alpha-ketolic steroids after the administration of the six "active" adrenal cortical steroids to individuals with severe adrenal cortical insufficiency. We have found, as has been demonstrated for other steroids (4), that the major alpha-ketolic metabolites result from the reduction in ring A of the steroid nucleus to the 3-alpha-hydroxy pregnane derivatives (the so-called "tetrahydro" form) and that the hydroxyl and ketone groups on carbon atom 11 are interconvertible for corticosterone and 11-dehydrocorticosterone as well as for hydrocortisone and cortisone.

METHODS

I. Terminology

Since steroid terminology is varied and the proper chemical names consume considerable space, the terms and abbreviations more commonly used in physiological research will be employed in this paper. Definitions and abbreviations are listed below.

Alpha-ketolic steroids: Pregnane derivatives having 20-keto and 21-hydroxy substituents (*i.e.*, alpha-ketol group).

Alpha-ketolic cortical hormones: The six alpha-ketolic steroids with physiological activity, also exhibiting an alpha-beta unsaturated ketone in ring A (Δ^4-pregnene type). These are:

F; Compound F (Kendall); hydrocortisone; Δ^4-pregnene-11-β, 17α, 21-triol-3, 20-dione.

E; Compound E (Kendall); cortisone; Δ^4-pregnene-17α, 21-diol-3, 11, 20-trione.

S; Compound S (Reichstein); 11-desoxy-17-hydroxycorticosterone; Δ^4-pregnene-17α, 21-diol-3, 20- dione.

B; Compound B (Kendall); corticosterone; Δ^4-pregnene-11-β, 21-diol-3, 20-dione.

A; Compound A (Kendall); 11-dehydrocorticosterone; Δ^4-pregnene-21-ol-3, 11, 20-trione.

DOC; 11-desoxycorticosterone; Δ^4-pregnene-21-ol-3, 20-dione.

These are listed in order of decreasing polarity. The acetate is indicated by the abbreviation ac.

Alpha-ketolic steroid metabolites (AKSM): Steroids with the alpha-ketolic side chain found in the urine. These are detectable by reaction of the alpha-ketol side chain with alkaline blue tetrazolium to produce a blue color. The unmodified abbreviation H_4 indicates reduction in ring A to form the 3α normal (*i.e.*, pregnane) reduction product. In addition to the alpha-ketolic cortical hormones, the major metabolites are:

H_4F; tetrahydro-hydrocortisone; pregnane-3α, 11β, 17α, 21-tetrol-20-one.

H_4E; tetrahydro-cortisone; pregnane-3α, 17α, 21-triol-11, 20-dione.

H_4S; tetrahydro-11-desoxy-17-hydroxycorticosterone; pregnane-3α, 17α, 21-triol-20-one.

H_4B; tetrahydro-corticosterone; pregnane-3α, 11β, 21-triol-20-one.

H_4A; tetrahydro-dehydrocorticosterone; pregnane-3α, 21-diol-11, 20-dione.

H_4DOC; tetrahydro-11-desoxycorticosterone; pregnane-3α, 21-diol-20-one.

Polarity: In the chromatograms, the most polar steroids (*i.e.*, those with the most hydroxyl and keto substituents) appear in the slowest moving fraction, X, and the least polar in the fastest moving fraction, Z. (See III, B Paper chromatographic and micro-analytic technics.)

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TABLE I

Urinary alpha-ketolic steroid metabolites: Identification studies *

Oral dose per day	Fraction and peak no.	Standard steroid † / Compound isolated	R_{DOC}† Toluene/P.G. (free)	R_{DOC}† Me. cyclohexane/P.G. (acetate)	U.V. absorb. 245 mμ	Porter-Silber	Maxima of spectra in H_2SO_4				Other
	X_1	H_4F	.0060	.19	0	+	260	330	415	515	
			.0054	.22	0	+	256	320	410	510	
	X_3	H_4E	.0085	.40	0	+	272	330	410	—	
			.0076	.40	0	+	270	325	410	—	
F_{AC}	X_{2a}	Unknown	.012‡	—	0	−	—	—	—	—	
400 mg.											
Exp. No. 1	X_3	F	.017	—	+	+	240	280	390	475	Fluorescence +
			.017	—	+	+	240	276	385	480	Fluorescence +
	X_{3a}	Unknown	.027‡	—	−	−	—	—	—	—	
	X_4	E	.041	.013	+	+	280	340	415	—	
			.036	.013	+	+	280	330	420	—	
	X_1	H_4F	.0060	.19	0	+	260	330	415	510	Infrared
			.0054	.25	0	+	260	330	410	510	Infrared
	X_2	H_4E	.0085	.40	0	+	270	335	410	—	Infrared
			.0088	.40	0	+	276	335	410	—	Infrared
E_{AC}	X_{2a}	Unknown	.012‡	—	0	−	—	—	—	—	
200 mg.											
Exp. No. 3	X_3	F	.017	—	+	+	240	280	390	475	Fluorescence +
			.020	—	+	+	240	280	385	475	Fluorescence +
	X_4	E	.041§	—	+	+	280	340	410	—	Infrared
			.041	—	+	+	276	340	415	—	Infrared
	Y_1	H_2E	.069‖	.036	0	+	—	—	—	—	
			.069	.037	0	+	—	—	—	—	
S_{AC}											
100 mg.	X_1	H_4S	.037	3.8	0	+	315	410	—	—	Infrared
Exp. No. 11			.045	4.3	0	+	315	410	—	—	Infrared

* *Comment concerning identification:* Isolated compounds showing similar infrared spectra or similar spectra in sulfuric acid to the standard steroid as well as similar running rates and other listed characteristics are considered to be positively identified. For absolute identification of the "tetrahydro" and "dihydro" compounds, infrared spectrometry is essential since the sulfuric acid absorption spectra of the C-3 and C-5 stereoisomers are similar.

The identity of the compounds not subjected to these spectrometric studies is less certain but it is believed that peak Y_1 (exp. No. 3) found after administration of 200 mg. of E_{ac} is probably H_2E since: 1) the R_{DOC} both as the free and acetylated form was similar to H_2E in the toluene-propylene glycol system and as the acetylated form in the methylcyclohexane-propylene glycol system, and 2) the compound did not absorb ultraviolet light at 245 mμ but did give a quantitatively positive Porter-Silber reaction. In addition H_2E has been identified by Schneider (27) in extracts of normal male urine. In the less extensively studied experiments (below the double line) the evidence concerning identity is largely inferential. See discussion concerning possibility of peaks Y_2 and Z_1 in experiments No. 12 and No. 5 being the allo isomers of H_4B and H_4A.

† The data for the standard steroid are written above those for the isolated steroid. (See Methods for significance of abbreviations.) R_{DOC} (see Methods) in toluene-propylene glycol run as the free steroid and R_{DOC} in methylcyclohexane-propylene glycol run as the acetate.

‡ Note similarity of R_{DOC} of peaks X_{2a} in experiments No. 1, No. 3, and No. 10; peaks X_{2a} in experiments No. 1 and No. 9; peaks Y_2 in experiments No. 12, No. 5, and No. 13; and peaks Z_2 in experiments No. 12 and No. 5.

§ R_{DOC} run as acetates in toluene-propylene glycol system was 0.31 for unknown and for standard of E_{ac}.

‖ R_{DOC} run as acetates in toluene-propylene glycol system was 0.47 for unknown and 0.51 for standard H_2E_{ac}.

¶ R_{DOC} run as free compound in methylcyclohexane-propylene glycol system was 0.034 (unknown Z_2 after administration of B, exp. No. 12).

** R_{DOC} run as free compound in methylcyclohexane-propylene glycol system was 0.07 for unknown and for standard compound A.

†† R_{DOC} run as free compound in methylcyclohexane-propylene glycol system was 0.036 (unknown Z_2 after administration of A_{ac}, exp. No. 5, compare with note ¶ above).

‡‡ Free compound in methylcyclohexane-propylene glycol system.

TABLE I—*Continued*

Oral dose per day	Fraction and peak no.	Standard steroid † / Compound isolated	Rdoc† Toluene/ P.G. (free)	Rdoc† Me. cyclohexane/ P.G. (acetate)	U.V. absorb. 245 mμ.	Porter-Silber	Maxima of spectra in H_2SO_4				Other
	Y_1	H_4B	.066	3.2	0	0	—	—	—	—	Infrared
			.069	3.2	0	0	—	—	—	—	Infrared
	Y_2	Allo-H_4B	.11	2.9	0	0	320	410	—	—	
			.10‡	3.0	0	0	310	420	—	—	
B_{ALC} 90 mg. Exp. No. 12	Y_3	H_4A	.14	5.6	0	0	290	355	415	—	
			.13	5.9	0	0	292	355	410	—	
	Z_1	B	.20	.15	+	0	—	—	—	—	Fluorescence +
			.20	.17	+	0	—	—	—	—	Fluorescence +
	Z_2	Unknown	.18‡	4.4¶	0	0	—	—	—	—	
	Z_3	A	.50	.52**	+	−	280	355	415	—	
			.50	.42	+	−	284	355	410	—	
	Y_1	H_4B	.066	3.2	0	0	310	415	—	—	
			.069	3.6	0	0	310	420	—	—	
	Y_2	Allo-H_4B	.11	2.9	0	0	—	—	—	—	
			.10‡	3.0	0	0	—	—	—	—	
A_{AC} 200 mg. Exp. No. 5	Y_3	H_4A	.14	5.6	0	0	290	350	415	—	
			.14	5.5	0	0	292	355	410	—	
	Z_1	B	.20	.15	+	0	284	320	375	450	Fluorescence +
			.21	.17	+	0	285	330	373	455	Fluorescence +
	Z_2	Unknown	.18‡	4.4††	0	0	292	355	410	—	
	Z_3	A	.50	.52	+	0	284	355	410	—	Infrared
			.55	.47	+	0	284	355	410	—	Infrared
	Y_1	H_4B	.066	3.2	0	−	315	410	—	—	
			.067	3.6	0	−	315	410	—	—	
A_{AC} 100 mg. Exp. No. 13	Y_2	Allo-H_4B	.11	2.9	0	−	—	—	—	—	
			.12‡	3.0	0	−	—	—	—	—	
	Y_3	H_4A	.14	—	0	−	290	355	415	—	Infrared
			.13	—	0	−	292	355	410	—	Infrared
	Z_1	H_4DOC	.31	17.0	0	0	—	—	—	—	Infrared
DOC 200 mg. Exp. No. 6			.33	18.0	0	0	300	360	430	—	Infrared
	Z_2	DOC	1.0	3.3	+	0	285	370	440	—	Anthrone +
			1.0	3.0	+	0	288	370	440	—	Anthrone +
Less extensively studied experiments											
	X_1	H_4F	.0060	—	0						
			.0062	—	0						
	X_2	H_4E	.0085	—	0						
			.0093	—	0						
F_{AC} 100 mg. Exp. No. 9	X_3	F	.017	—	+						Fluorescence +
			.017	—	+						Fluorescence +
	X_{3a}	Unknown	.029‡	—	0						
	X_4	E	.041	—	+						
			.041	—	+						

TABLE I—*Continued*

Oral dose per day	Fraction and peak no.	Standard steroid † / Compound isolated	R_{DOC}† Toluene/P.G. (free)	R_{DOC}† Me. cyclohexane/P.G. (acetate)	U.V. absorb. 245 mμ. Porter-Silber	Maxima of spectra in H_2SO_4	Other
	X_1	H_4F	.0060	—	0		
			.0056	—	0		
	X_2	H_4E	.0085	—	0		
			.0085	—	0		
E_{AC} 100 mg. Exp. No. 10	X_{2a}	Unknown	.013‡	—	–		
	X_3	F	.017	—	+		Fluorescence +
			.017	—	+		Fluorescence +
	X_4	E	.041	.013	+		
			.041	.012	+		
	Z_1	H_4DOC	—	—	0		
DOC_{AC} 100 mg. Exp. No. 14			—	—	0		
	Z_2	DOC	—	1.0‡‡	+		Anthrone +
			—	.98	+		Anthrone +

R_{DOC}: Mobility of compound compared to 11-desoxycorticosterone. (See Identification Technics.)

"Group reaction": Reactions given by a number of steroids having common chemical properties which depend primarily upon substituent groups. These include the reaction with blue tetrazolium of the alpha-ketolic side chain, reduction of phosphomolybdic acid by material extracted after acid hydrolysis (Ac. R-L), and that extractable after hydrolysis with glucuronidase and acid (G'ase R-L) (so-called reducing lipids, corticoids, lipid-soluble material with reducing properties due to the alpha-ketolic side chain and/or unsaturated ketone in ring A), and the total neutral 17-ketosteroids (17-KS), *i.e.*, those substances giving the Zimmerman reaction in neutral urinary extracts not subjected to separation with Girard's reagent. The reaction is primarily due to the presence of a keto group at carbon atom 17, but keto groups at other positions may react to some extent.

Identification: When used without modification this term indicates that the isolated alpha-ketolic steroid has been subjected to spectrometry in sulfuric acid or infrared spectrometry, or both, as well as the following tests (with a few exceptions):

1. Reaction with alkaline blue tetrazolium.
2. R_{DOC} in two or more solvent systems as free and acetylated forms.
3. Ultraviolet light absorption at 245 mμ.
4. Phenylhydrazine reaction.
5. And other appropriate tests as indicated in Table I and under chemical methods.

It is emphasized that so-called identification based only on position on the chromatogram and one or two reactions is not considered adequate.

II. Experimental Procedures

A. Subjects

Case No. 1. Severe secondary adrenal cortical insufficiency. Subject AL., a 30-year-old Negro housewife, was studied over the period November 25, 1951 to February 18, 1953. She exhibited classical evidence of severe insufficiency of the anterior pituitary for one and one-half years prior to starting the study. This was presumably due to a non-functioning tumor as indicated by enlargement of sella turcica by x-ray. Visual field studies showed no evidence of pressure on the optic nerves. Throughout the period of special study, she was maintained in fairly good condition by androgens and estrogens which were stopped for 48 hours or more prior to administration of the compound to be studied.

Case No. 2. An adrenalectomized, orchidectomized man. Subject SCH. had his testes removed for prostatic carcinoma three years prior to his bilateral adrenalectomy at the age of 65 years. The studies were made one month after adrenalectomy when he was admitted to the hospital on December 9, 1952, with signs of insufficiency of adrenal cortical replacement therapy.

Case No. 3. Severe primary adrenal cortical insufficiency. Subject JO., a 55-year-old male scientist, had suffered from Addison's disease for five years prior to the studies which were made from the period November 19, 1951 to February 18, 1952. During this period, no special therapy was given or needed since he had a subcutaneous pellet of 11-desoxycorticosterone acetate implanted on July 21, 1951. Shortly after completion of the studies, he showed mild signs of possible adrenal insufficiency and additional therapy was given. Chromatograms are *not* illustrated but showed patterns after F, E, S, and DOC similar to those shown in Figures 1 and 2.

B. Steroids[2]

The commercially available steroids (hydrocortisone, cortisone, and 11-desoxycorticosterone) which had been donated to us were administered without chemical examination. The other alpha-ketolic cortical hormones, *i.e.*, corticosterone (B), 11-dehydrocorticosterone (A) and 11-desoxy-17-hydroxycorticosterone (S), were subjected to chromatography and found to be essentially pure as determined by this method. Steroids were administered as the acetate with the exception of corticosterone (B) which was given in the free form. The compounds were administered within the first hour of the metabolic day with the exception of cortisone acetate (in exp. No. 13) which was given in a dosage of 50 mg. every six hours for three and one-half days.

C. Urine collection

Urine was collected for 24-hour periods in brown glass bottles containing approximately 10 ml. of chloroform and kept in the ice box during and after the collection period. Creatinine was determined as a gross check on the accuracy of collections.

III. Chemical Procedures

A. Hydrolysis and extraction

Within a day after completing the collection of urine, the specimens were brought to pH 4.5 with a mixture of sodium acetate and acetic acid so that the acetate concentration was 0.1 normal, and incubated for 48 hours at 38° with 100 Fishman units per ml. of Viobin beef spleen β-glucuronidase using a few drops of $CHCl_3$ as preservative. Glucuronidase incubated with distilled H_2O under the same conditions gave no appreciable ultraviolet absorbing or blue tetrazolium reacting material.

The urines were then adjusted to pH 1 and extracted for one hour with an equal volume of $CHCl_3$ on an oscillating shaker, then washed with cold 0.1 N sodium hydroxide and water and evaporated to dryness in vacuo after drying over sodium sulfate.

Comment. Early in this study, the use of β-glucuronidase for hydrolyzing steroid conjugates was investigated. Neutral reducing lipid-soluble substances in urine were compared after hydrolysis at pH 1 and after glucuronidase hydrolysis in a series of normal individuals and patients. In most cases, it was found that values approximating the plateau value were obtained by using the procedure outlined above. These values varied from 2 to 13 times the values obtained after extraction at pH 1.

The effect upon the yield of alpha-ketolic steroids from aliquots of a large sample of urine was studied under the following conditions: 1) Extraction with chloroform at pH 1 at room temperature for one hour; 2) extraction with chloroform after: (a) glucuronidase hydrolysis as indicated above, followed by (b) re-extraction with chloroform at pH 1 for one hour; and 3) hydrolysis with glucuronidase as indicated above followed by hydrolysis at pH 1 during a single extraction with chloroform for one hour. The yield of alpha-ketolic steroids with procedure No. 1 was 0.1 mg. per 24 hours; with procedure No. 2a, the yield was 1.1 mg. per 24 hours; with No. 2b there was a further yield of 0.3 mg. per 24 hours and with procedure No. 3 a yield of 1.4 mg. per 24 hours. Measurement of the reducing lipids gave similar results. As a result of these and other studies and considering the cost of the β-glucuronidase for the large volumes of urine hydrolyzed, procedure No. 3 was adopted as yielding most of the alpha-ketols present. More recent work has indicated that a pH of 4.8 is optimum for hydrolysis (5). It has also been demonstrated that hydrolysis is not complete at the plateau since addition of more glucuronidase and continued incubation, particularly after extraction of the corticoids released, gives still more steroid (6).

Re-extraction with an equal volume of $CHCl_3$ indicated that about 85 per cent of the total of the two extractions was obtained on the first extraction. This was measured by determination of neutral reducing lipids and also by calculations from the areas of the alpha-ketol peaks on the paper chromatograms (see below).

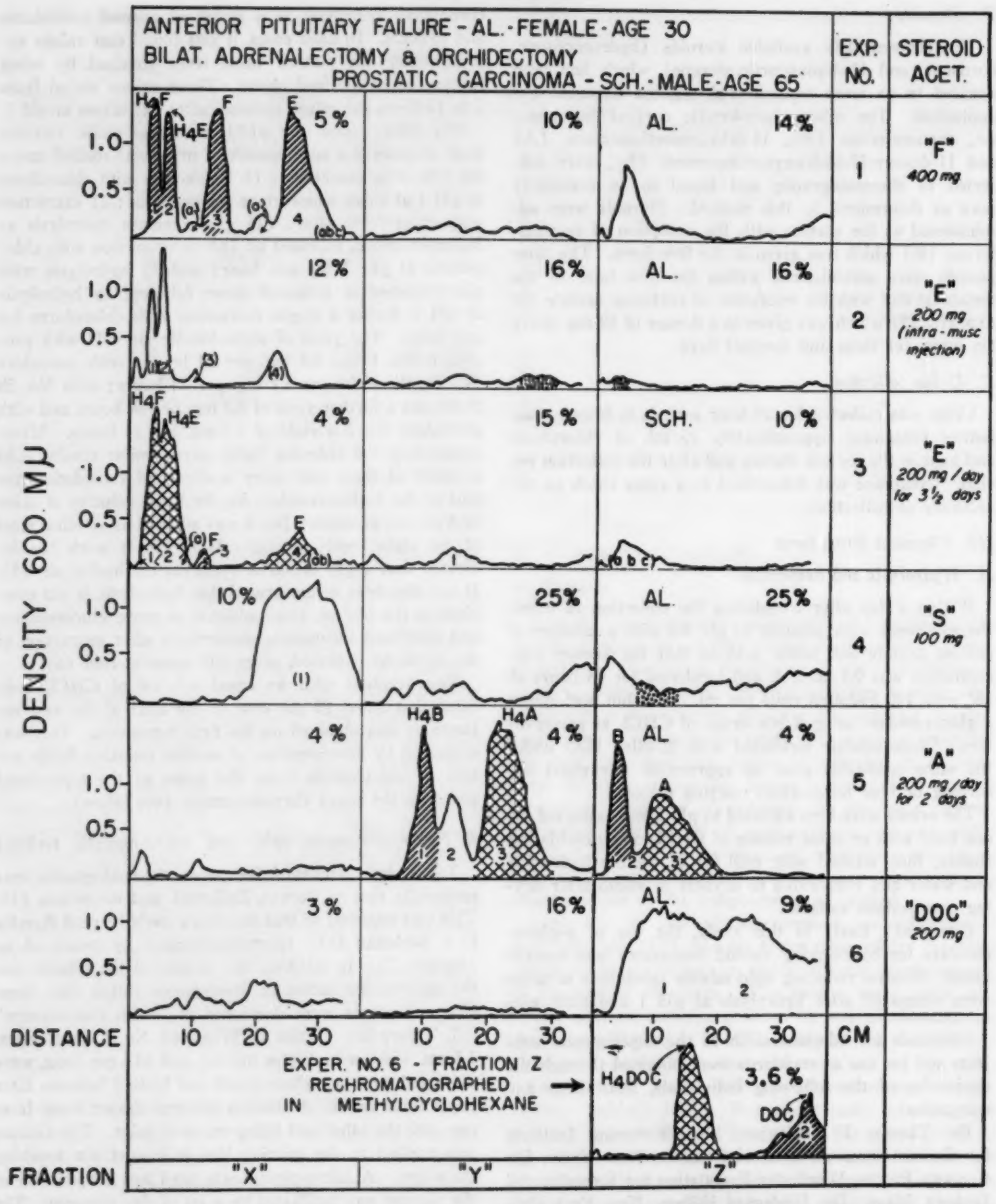
B. Paper chromatographic and micro-analytic technics

The technic employed for paper chromatography was essentially that of Burton, Zaffaroni, and Keutmann (1). This was modified so that the strips could be read directly in a Beckman D.U. spectrophotometer by means of an adaptor (7). In addition, the volume of the effluent was the determining factor in development rather than time. Chromatograms were developed at "room temperature."

1. *Separation.* Strips of Whatman No. 1 filter paper, 1.3 cm. wide, or multiples thereof, and 44.5 cm. long, were saturated with propylene glycol and blotted between filter paper three times. A starting line was drawn 9 cm. from one end, the other end being cut to a point. The extract was applied to the starting line in a spot not touching the edges. A micro-pipette was used and evaporation of the solvent was facilitated by a jet of dry nitrogen. The chromatograms were then developed essentially as described by Burton, Zaffaroni, and Keutmann (1). The strips were then removed and dried at room temperature. The first (most polar) fraction, X, is found on the first strip after collection of 14 to 15 ml. of effluent per 1.3 cm. width of strip. The running time varied from 72 to 96 hours. The effluent of the most polar fraction is

[2] Steroids for administration to the experimental subjects and for use as standards were obtained through the generosity of the following individuals, institutions and companies:

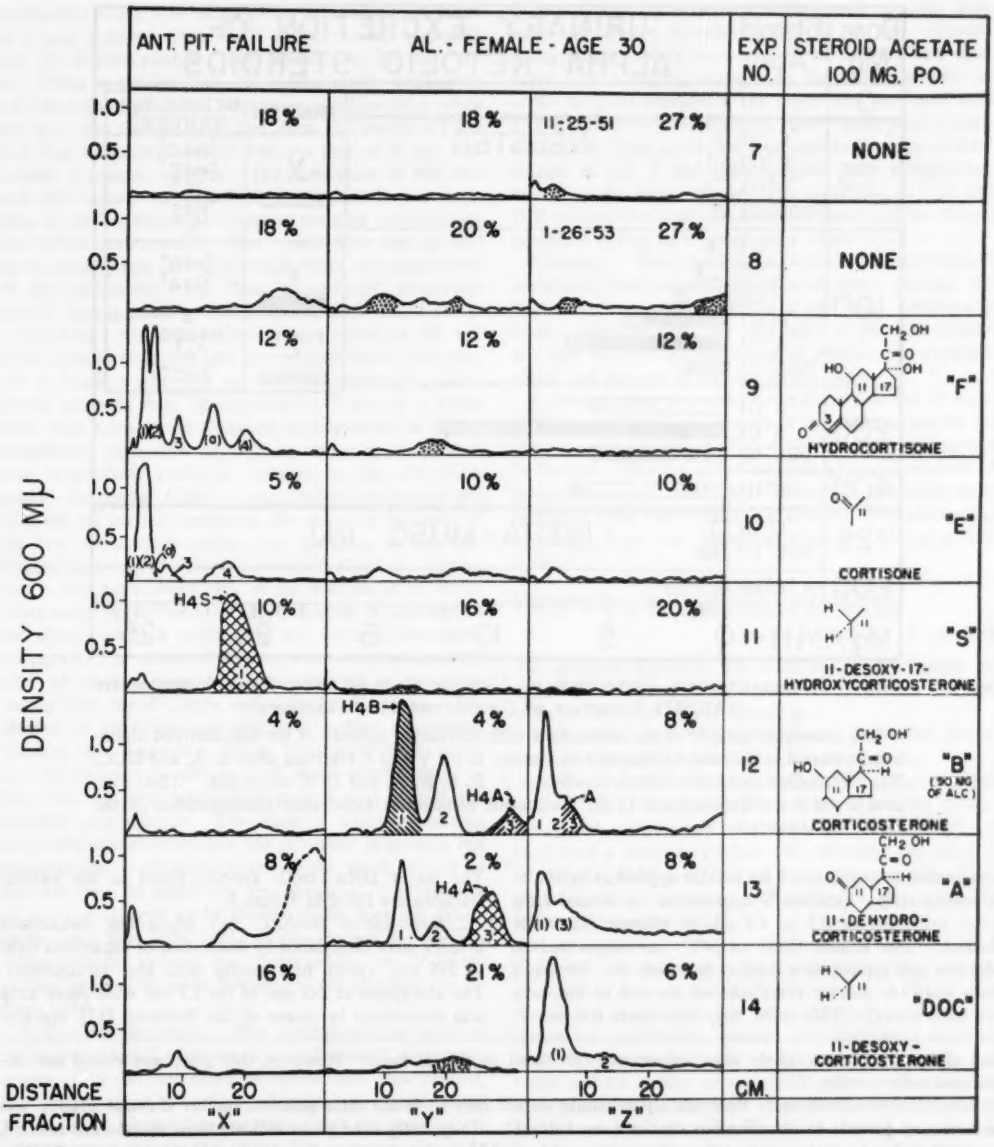
Dr. Thomas F. Gallagher, Sloan-Kettering Institute for Cancer Research, Memorial Center, New York; Dr. Gregory Pincus, Worcester Foundation for Experimental Biology, Mass.; Dr. Hildegard Wilson, New York University, New York; Ciba Pharmaceutical Products, Inc., Dr. Ernst Oppenheimer and F. E. Houghton; Eli Lilly and Company, Dr. O. M. Helmer; Merck and Company, Drs. Elmer Alpert, Lewis H. Sarett, and Karl Pfister; Schering Corporation, Robert E. Waterman; G. D. Searle and Company, Dr. Irwin C. Winter; and The Upjohn Company, Dr. William J. Haines.



FIGS. 1 AND 2. PRELIMINARY CHROMATOGRAMS OF ALPHA-KETOLIC STEROID METABOLITES

Figures 1 and 2 illustrate the major alpha-ketolic chromatographic peaks found in the urine extracts following oral (or intramuscular—esp. No. 2) administration of various doses of active cortical hormones to subjects with markedly reduced or absent adrenocortical function. Experiments No. 7 and No. 8 (Figure 2) show the control studies.

The formulas given on Figure 2 are those of the free steroids. The corresponding acetates were administered in all cases except compound B.



These "preliminary" chromatograms were developed in the toluene-propylene glycol system. After drying, they were sprayed with blue tetrazolium and the density of the blue color produced by reaction with the alpha-ketols was then determined in a Beckman D.U. spectrophotometer at 600 mμ. The *density* is charted as the ordinate in relation to the *distance* from the "starting line." The most polar (X), "mid-polar" (Y), and least polar (Z) *fractions* are charted. The *percentage figures* in each fraction indicate the proportion of the 24-hour urine extract used on the chromatogram. The *dotted areas* indicate zones of atypical color development. The dashed line for the last peak in the X fraction, exp. No. 13, indicates that this peak is usually found in fraction Y, but the development of fraction X was

(*Continued on page 292*)

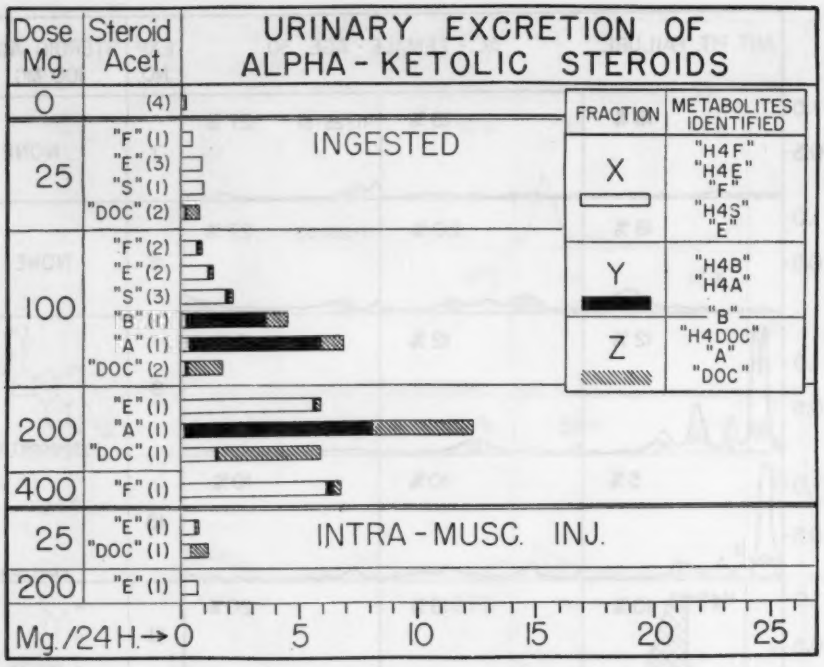


FIG. 3. SEMIQUANTITATIVE ESTIMATION OF URINARY ALPHA-KETOLIC STEROID METABOLITE (AKSM) EXCRETION BY CHROMATOGRAPHIC FRACTIONS

The decreasing polarity of the metabolites with decreasing polarity of the administered alpha-ketolic steroid is indicated by the marked increase in the Y and Z fractions after B, A, and DOC. The metabolites found after administration of F, E, S, B, A, and DOC are listed. (See Figures 2 and 3 and Tables I and II for the specific metabolites found after administration of the individual steroids.)

evaporated to dryness and the residue applied as before to a second strip. Fraction Y appears on the second strip after collection of 3.5 to 4.0 ml. of effluent (15 to 24 hours). The effluent from strip Y was evaporated to dryness and applied to a third strip which was developed only until the solvent front reached the end of the strip (3 to 6 hours). This third strip represents fraction Z. The major alpha-ketolic steroids found in the various fractions are listed in Figure 3.

2. *Detection of steroids.* (a) Alpha-beta unsaturated ketones were determined by absorption of ultraviolet light at 245 mμ. (prior to spraying with blue tetrazolium). The absorption at 245 mμ. of the 1.3 cm. wide paper strip was determined by means of the Beckman D.U. spectro-

not allowed to proceed to the standard amount of effluent in this instance. However, this peak was eluted and developed with fraction Y.

Identification: Since more than one alpha-ketolic steroid may be in the same position, further chromatography was a necessary prelude to identification studies (see Table I). *Diagonally lined* peaks indicate those metabolites isolated, purified and subjected to infrared spectrometric analysis \\\ or spectrometry in sulfuric acid /// or both ▨. *These peaks are considered as identified as indicated by the abbreviations (see Methods) above the peaks.* Other *numbered peaks, without parentheses* were re-chromatographed with a standard and R_{DOC} determined in one or more solvent systems as the free or acetylated forms and have been subjected to special tests as indicated in Table I. The *peaks with numbers in parentheses* have been numbered (without re-chromatographing) and R_{DOC} calculated on the basis of position relative to a peak of known R_{DOC} (on the strip).

Peaks with the same number in similar fractions after administering the same compound are probably due to the same metabolite, although positive identification has been made only in those indicated by the diagonal lines. The same number after feeding of non-identical steroids does not indicate the two or more peaks so numbered to be identical in composition. Less completely studied peaks have been indicated by the letters a, b, *etc.*

photometer fitted with an adaptor. Readings were taken at 7 mm. intervals (or less if optical density changed) and the results plotted. The readings at 245 mμ. are *not* shown in the figures. (b) Alpha-ketolic compounds were then detected by the production of a blue color when the strip was sprayed with two parts of aqueous 0.2 per cent blue tetrazolium mixed with one part of 10 per cent sodium hydroxide solution. The absorption at 600 mμ. was determined, as described above, within one hour after air drying the strips following spraying with alkaline blue tetrazolium solution. The results were then plotted on the same graph so that a visual record was maintained of the "ultraviolet" and "blue tetrazolium" absorption curves (peaks) which demonstrated their relationship.

Comment. Although ultraviolet absorption at 245 mμ. is not specific for alpha-beta unsaturated ketonic steroids, nor is reaction with blue tetrazolium confined to alpha-ketolic steroids, these observations have proved a useful first step toward identification and provide a rough quantitative estimation as well as an exact location of blue tetrazolium peaks in relation to the ultraviolet peaks. In several series of quantitative chromatograms with the six cortical hormones, the ratios of the areas of the blue tetrazolium peaks, after spraying to the corresponding areas at 245 mμ., averaged about .7. Therefore a blue tetrazolium peak at the position of an ultraviolet peak having an area approximately 0.7 of that of the ultraviolet peak which does not change when chromatographed in other solvents or when chromatographed after acetylation suggests the presence of a compound having both the Δ^4-3-keto and alpha-ketol groups. The absence of ultraviolet absorption in a blue tetrazolium reacting zone suggests a "di- or tetrahydro" alpha-ketol. An ultraviolet peak with an area much greater than, or less than, the blue tetrazolium stained area implies the presence of a mixture. The shape of either curve may also indicate that more than one substance is present, but irregularities in distribution across the paper, visible to the eye, must be taken into consideration. The absence of peaks as determined by these technics does not necessarily mean that other types of steroids are not present, since steroids without the alpha-ketolic or Δ^4-3-keto groups, as well as other substances, are not detected by these tests.

3. *Re-chromatography.* The chromatograms pictured in Figures 1 and 2 represent the "preliminary" chromatograms. For further characterization and identification, appropriate zones of unsprayed strips were selected on the basis of ultraviolet absorption and the position of blue zones on the parallel strip after spraying with blue tetrazolium solution. These zones were eluted with methanol and re-chromatographed in the appropriate system with the required standards as the free compound and after acetylation. Acetates usually required the methylcyclohexane-propylene glycol system. Acetylation and subsequent determination of R_{DOC} afforded evidence concerning substituent groups. The use of such procedures often resulted in wide separation of two compounds with similar R_{DOC} in the toluene-propylene glycol system. It also frequently separated alpha-ketolic steroids from other ultraviolet absorbing material in the extracts. Some peaks, notably those in fraction Z, were best separated with methylcyclohexane as the mobile phase. In order to speed movement, the paper was saturated with 1:1 methanol-propylene glycol rather than pure propylene glycol. This procedure was used in the case of compounds A and B and their acetates with methylcyclohexane as the mobile phase. For separation of H_4F and H_4E toluene was used as the mobile phase and the diluted propylene glycol as the stationary phase.

Comment. Reproducibility—Because of temperature variations, displacement effects and other factors, the R_{DOC} may vary approximately 10 per cent; occasionally more. Absolute rates per unit time or per unit volume are also affected by variations in amount of propylene glycol and amount of extract on the paper.

4. Identification procedures. a. Determination of R_{DOC}. Relative mobility was expressed as distance moved on the strip with respect to DOC taken as unity as described by Burton, Zaffaroni, and Keutmann (1). Compounds of known R_{DOC} were used where indicated. Distances were measured from the starting line to point of maximum absorption. R_{DOC} was calculated from the following formula:

$$\text{Unknown } R_{DOC} = \frac{\text{Distance moved by unknown}}{\text{Distance moved by standard}} \times R_{DOC} \text{ of standard}$$

For greatest accuracy, the substances should move an appreciable distance from the starting line but not too close to the lower end of the strip.

b. Reaction with blue tetrazolium. Described above.

c. Absorption of ultraviolet. Described above.

d. Reaction with phenylhydrazine. The Porter-Silber method (8) for determination of F and other 17-hydroxylated alpha-ketols was used. More recently, we have used a modified method (*i.e.*, allowing the reaction mixture to stand overnight at room temperature). This gives identical extinction coefficients for all tested 17-hydroxylated alpha-ketols.

e. Absorption spectra in sulfuric acid. The method of Zaffaroni (2) was employed, using micro-cells and approximately 8 to 10 μg. of the steroid. Special preparation of the paper strips and care with glassware was necessary for satisfactory results. All equipment must be washed with chromic acid and thoroughly rinsed with water and the elution solvent. All solvents are redistilled and tested for absorption characteristics. The paper strips used in final purification were washed in a beaker for three days in 3 per cent acetic acid, one day with water, and three days in methanol, with change of the solvents several times during each washing. After elution, the eluate was filtered through fritted glass to eliminate paper fibre. Using these precautions, satisfactory spectra can be obtained in most instances using pure sulfuric acid as a blank.

Comment. The absorption spectra in sulfuric acid appears to be a valuable tool in the identification of C-21 steroids. However, it is not always possible to differ-

entiate stereo-isomers, nor in all cases can the saturated steroids be distinguished from those containing an isolated double bond (2, 9). In our experience, the absorption spectra for the alpha-ketolic steroids tested by us or reported by others (2, 9) are distinctive (except for some stereo-isomers) when extinction coefficients as well as maxima and minima are compared. Absorption curves for some standards and isolated compounds are shown in Figure 4.

f. Infrared spectrometry. Infrared spectrometry (10) was done if sufficient material was isolated. The analyses were made at the Sloan-Kettering Institute for Cancer Research, Memorial Center, New York City, through the courtesy of Dr. Thomas F. Gallagher. Steroids isolated by paper chromatography are frequently not sufficiently pure for infrared spectrometry. Therefore, they were purified further as their acetates on silica gel columns.

g. Miscellaneous procedures. 1) In general, the isolated substances which did not show ultraviolet absorption were subjected to the modified Pettenkofer reaction (11) using dehydroepiandrosterone as the reference standard. None of the isolated substances have shown a positive reaction in this test.

2) The quantitative anthrone test was applied to the substances isolated and suspected to be DOC (12).

3) The fluorescence of compounds F and B in sulfuric acid has been considered characteristic for these substances (13). A quantitative technic, modified by omission of the initial heating period, from that of Engel, Slaunwhite, Carter, and Nathanson (14) for estrogens was used, employing the primary and secondary filters for

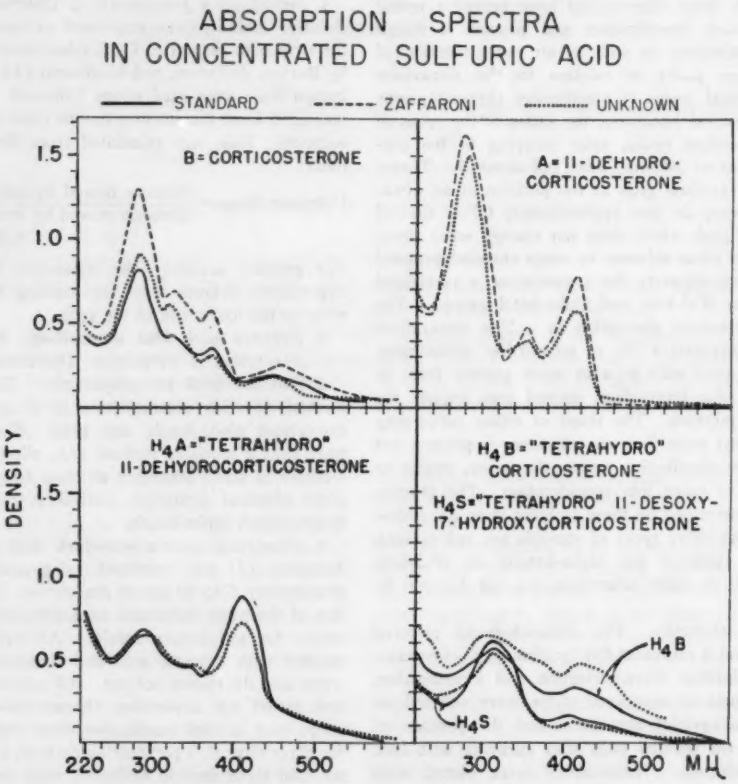


FIG. 4. ABSORPTION SPECTRA IN SULFURIC ACID

The experiment number and peak number indicating the source of the unknowns illustrated in this figure are as follows: B—exp. No. 5, peak Z_1; A—exp. No. 12, peak Z_2; H_4A—exp. No. 13, peak Y_2; H_4B—exp. No. 5, peak Y_1, and H_4S—exp. No. 11, peak X_2. Further identification data concerning these steroids are given in Table I and chromatograms are shown in Figures 1 and 2. The similarity of H_4B and H_4S in regard to the region of maximum absorption and their dissimilarity in regard to relative density is illustrated.

the B_3 determination furnished with the Lumetron fluorimeter. It was found that none of the other active cortical hormones nor their normal 3-α hydroxy derivatives show more than one-fifteenth the fluorescence of F. The fluorescence relative to hydrocortisone (F), taken as 100 per cent, of the neutral steroids tested is as follows: Corticosterone (B) 150 per cent; Δ^4-pregnene-17α, 20, 21-triol-3-one 50 per cent; Δ^4-pregnene-11β, 17α, 20, 21-tetrol-3-one 25 per cent; Δ^4-androstene-17α-ol-3-one ("cis"-testosterone) 25 per cent; and Δ^4-pregnene-17β, 20, 21-triol-3-one 17 per cent. The following steroids gave less than 7 per cent: H_2F acetate; H_4F diacetate; E; H_4E diacetate; H_4B; A; H_4A; S; H_4S acetate; DOC; H_4DOC; testosterone-17β; progesterone; pregnane-3α, 20α-diol; and Δ^5-pregnene-3β, 21-diol-20-one.

h. Approximation of AKSM excretion. Studies with known compounds indicate that the area under the curves of density at 600 mμ. (as read directly from the paper) plotted against distance on the chromatogram appear to be directly related to the quantity of steroid between the values of 10 and 60 micrograms. The range of variation from the mean was $\pm$ 30 per cent. An approximation of the amount in the extract of the 24-hour urine collection can be obtained from this estimate and the proportion of the 24-hour urine extract used on the chromatographic strip. This method can be used to indicate total AKSM as well as the estimation of a particular alpha-ketolic steroid metabolite.

C. Group reactions

The alpha-ketolic steroid metabolite (AKSF) excretion was approximated as described above. Lipid-soluble reducing substances (G'ase. R-L and Acid R-L) were determined, after both methods of hydrolysis, by the method of Heard, Sobel, and Venning (15). The total neutral 17-ketosteroids were measured by a modification of the Holtorff-Koch technic (16) on aliquots extracted with carbon-tetrachloride without subjecting the material to Girard separation.

Comment. The lack of specificity of the reducing properties of lipid-soluble extracts is well recognized as is the non-ketonic and non-steroidal nature of some of the material measured as "total neutral 17-ketosteroids," *i.e.*, not subjected to Girard separation. In addition, keto groups at positions other than carbon atom 17 contribute to the total.

RESULTS

Results are presented as follows: 1) control studies, 2) identification of alpha-ketolic steroid metabolites following the administration of known compounds, 3) semiquantitative estimates of identified alpha-ketolic steroid metabolites, 4) the chromatographic pattern of the major urinary alpha-ketolic steroids, and 5) the effects of administration of known alpha-ketolic steroids on group reactions. In the figures and tables, the results are grouped in order of decreasing polarity according to the specific compound administered.

1. Control studies

The control chromatograms of the extracts of urine from subject AL. collected during periods when no steroids were administered are shown in Figure 2. These indicate the small amount of blue tetrazolium reacting material excreted during the control periods at the beginning and end of the experiments. Large amounts of the 24-hour extract were used on the chromatograms. The nature of the substance reacting with blue tetrazolium in the X fraction of the control study (exp. No. 8) is unknown. No urine was collected in the case of patient SCH. in the absence of therapy. Since this man was adrenalectomized and orchidectomized, it is presumed that little alpha-ketolic steroid would be contributed by the subject. Control chromatograms in patient JO. (not shown) and three other patients with adrenal insufficiency have demonstrated the small amount of AKSM excreted in the urine of individuals with severe adrenal cortical insufficiency. Details concerning case histories are in the section on Methods.

2. Identification of alpha-ketolic steroid metabolites

Figures 1 and 2 show the preliminary chromatograms and Table I lists the results of the identification studies on the alpha-ketolic steroid metabolites (AKSM) extracted from the urine after administration of the six alpha-ketolic cortical hormones. Only those isolated materials subjected to infrared spectrometry or spectrometry in sulfuric acid (or both) in addition to the other studies are considered to be "identified." These studies indicate that the major alpha-ketolic metabolite of all six alpha-ketolic steroids is the so-called "tetrahydro" form. Infrared spectrometry indicated that the 3-α hydroxy pregnane configuration was present. (Suggestive evidence for the presence of allo-H_4B after feeding B or A will be considered in the discussion.) The "tetrahydro" derivatives of both F and E after feeding E have been identified by infrared spectrometry as well as other tests. The isolation of tetrahydro derivatives of S, A, B, and DOC after feeding these compounds has also been confirmed by infrared spec-

trometry. The standard samples of these compounds for use in our laboratory were obtained through the courtesy of the following: Pregnane-3α, 17α, 21-triol-20-one, (H_4S), Dr. Thomas F. Gallagher (17); Pregnane-3α, 21-diol-20-one, (H_4DOC), Dr. Thomas F. Gallagher (unpublished synthesis, Dr. A. L. Kemp); and from Dr. Karl Pfister, Merck & Co., Pregnane-3α, 11β, 21-triol-20-one, (H_4B) (unpublished synthesis, Dr. David Taub), and Pregnane-3α, 21-diol-11, 20-dione, (H_4A). The tetrahydro forms identified by various procedures including spectrometry in sulfuric acid but not infrared (due to lack of material) are as follows: tetrahydro F after the administration of compound F, tetrahydro A after feeding compound B, tetrahydro B after feeding compound A, and allo tetrahydro B after feeding B.

The interconvertibility of the hydroxyl and keto substituents at carbon atom 11 of corticosterone (B) and 11-dehydrocorticosterone (A) as well as hydrocortisone (F) and cortisone (E) has been demonstrated by the isolation of A and H_4A after feeding B; B and H_4B after feeding A; as well as E and H_4E after feeding F; and F and H_4F after feeding E (Figures 1 and 2 and Table I).

Comment: Others have previously demonstrated the interconvertibility in the human subject of these substituents for hydrocortisone (18), cortisone (19–21) and adrenosterone (22). As indicated above, all of the six administered alpha-ketolic steroids have been shown to be reduced in ring A. Reduction of the alpha-beta unsaturated ketone in ring A by the human subject has previously been demonstrated for many steroids, particularly those in the C-19 series such as testosterone and the progesterone series (4) as well as for cortisone (19–21) and hydrocortisone (18). These findings have recently been summarized by Dorfman and Ungar (4). The reduction of ring A in DOC (23) and compound A (24) with concomitant reduction of the alpha-ketolic side chain has also been described. The tetrahydro forms of compounds S, B, A and DOC do not appear to have been described previously as urinary metabolites of their respective unsaturated parent compounds, nor has the formation of corticosterone from 11-dehydrocorticosterone and the reverse reaction been described previously.

3. Estimation of identified alpha-ketolic steroid metabolites

Table II lists the amounts of the identified alpha-ketolic metabolites that have been found in the urine after administration of the six compounds. The tetrahydro forms of the administered steroids F, E, B, and A are greater in amount than the tetrahydro forms of their conversion products, *i.e.*, those resulting from interconversion of the hydroxyl and keto groups at carbon atom 11.

Comment: The relatively high excretion of compound F (after 400 mg. as compared with that after 100 mg. of Fac) in relationship to its metabolites suggests the possibility that the capacity of the metabolic systems responsible for the reduction of ring A and the interconversion at carbon atom 11 may have been overburdened. A similar pattern may be noted (by giving due consideration to the per cent of the 24-hour extract utilized) in Figures 1 and 2 by comparing those experiments in which 100 and 200 mg. of A and 100 and 200

TABLE II

Semiquantitative estimation of identified urinary alpha-ketolic steroid metabolites (mg./24 hr.) *

Steroid acetate adm.	Dose mg./24 hr.	Exp. no.	H_4F	H_4E	F	E	H_4S	H_4B	H_4A	B	A	H_4DOC	DOC
F	400	1	0.9	0.8	1.5	0.1	—	—	—	—	—	—	—
E	200	3	1.2	3.4	0.2	0.6	—	—	—	—	—	—	—
S	100	11	—	—	—	—	0.9	—	—	—	—	—	—
B (As alc.)	90	12	—	—	—	—	—	2.2	0.5	0.62†	0.1	—	—
A	200	5	—	—	—	—	—	2.0	4.5	1.0	1.1	—	—
DOC	200	6	—	—	—	—	—	—	—	—	—	2.7	1.5

* Extractable by method used (hydrolysis with 100 units glucuronidase per ml. followed by extraction with $CHCl_3$ at pH_1). Identified by infra-red spectrometry and/or spectroscopy in sulfuric acid and other procedures listed in Table I.
† Partial identification—see Table I.

mg. of DOC were administered. The relative proportion of the material at the position of the parent compound is greater in those experiments in which 200 mg. was administered.

4. The chromatographic pattern of the major urinary alpha-ketolic steroids

Comparison of Figures 1 and 2 shows that there appears to be a similarity of pattern when the same steroid is administered although some differences are also present.

Comment: The decreasing polarity of the major alpha-ketolic metabolites with decreasing polarity of the administered steroid is obvious from inspection of Figures 1 and 2 as is the similarity of the pattern after administration of F_{ac} and E_{ac} and after B and A_{ac} administration. The characteristic patterns have been obtained in subject AL. (case No. 1) after doses of less than 100 mg. as well as in subject JO. (case No. 3) and other subjects (not shown).

5. Group Reactions

A. Semiquantitative estimation of total alpha-ketolic steroid metabolites (AKSM): The AKSM excretion was calculated from the areas of the peaks in each fraction of the chromatograms and the proportion of the 24-hour urine extract chromatographed (see methods). Results by chromatographic fractions are charted in Figure 3 and the total AKSM are tabulated in relationship to other group reactions in Table III. Following administration of 25 mg. of the alpha-ketolic cortical hormone, urinary AKSM increased above base-line values but the administration of 200 mg. or more seems necessary to produce marked increases in excretion. The AKSM excretion following the administration of compound F is less at comparable dose levels than for the other steroids administered. This corresponds to the finding of lesser amounts of G'ase R-L in the urine in these experiments.

B. Neutral reducing lipids extractable after glucuronidase plus acid hydrolysis (G'ase R-L) and after acid hydrolysis alone (Acid R-L): Table III shows the urinary excretion of phosphomolybdic acid "reducing lipids" after the two methods of hydrolysis. The 25 mg. dose level had relatively little or no effect. In general, the acid R-L is only slightly (but significantly) increased by the administration of 100 mg. or more of the alpha-ketolic steroids by mouth, but marked increases in the absolute amounts of G'ase R-L occurred.

Comment: As with the AKSM, the G'ase R-L excretion tended to increase with increasing doses of the administered steroid, although there was some variability. The values for Acid R-L and most of those for G'ase R-L (after the feeding of steroids) are within the limits found for normal individuals by the same methods (unpublished).

Thorn and his co-workers (25) have reviewed the effect upon group reactions of administration of adrenocortical hormones. Increased excretion of neutral reducing lipids following the administration of hydrocortisone and cortisone is a well recognized effect. Our finding of increased excretion of G'ase R-L after compounds S, B, A, and DOC should be expected, but apparently there is relatively little in the literature concerning it.

C. Ratio G'ase R-L to AKSM: The lowest ratio G'ase R-L to AKSM is found with the large doses of administered steroid, and the highest ratios are found in the control studies (Table III).

Comment: This is additional evidence that much of the material measured as R-L in urine after glucuronidase (and also after acid) hydrolysis is non-steroidal. However, some of the differences in the two values may well be explained by the fact that alpha-beta unsaturated ketones also reduce phosphomolybdic acid, although not to the same extent as the alpha-ketols.

D. Total neutral 17-ketosteroids: Table III shows the total neutral 17-ketosteroid excretion following the administration of the six alpha-ketolic adrenocortical hormones. There was considerable variability in the results. In general, amounts greater than the highest control values were excreted, although none of the values are greater than those found for normal young adults.

Comment: The finding of increased 17-ketosteroid excretion (by a "group reaction" technic) after the administration of those alpha-ketolic steroids with a hydroxyl function at carbon atom 17 (F, E, and S) is to be expected and agrees with other reports (25). The isolation and identification of 11-oxygenated 17-ketosteroids following the administration of cortisone (19, 25) and hy-

TABLE III

Urinary excretion of metabolites measured by group reactions *

Oral dose (As acetate) mg.	AKSM† mg./24 hr. JO.	AKSM† mg./24 hr. AL.	G'ase R-L† mg./24 hr. JO.	G'ase R-L† mg./24 hr. AL.	Ratio† G'ase R-L/AKSM JO.	Ratio† G'ase R-L/AKSM AL.	Acid R-L† mg./24 hr. JO.	Acid R-L† mg./24 hr. AL.	17-KS† mg./24 hr. JO.	17-KS† mg./24 hr. AL.
No medication										
0	—	<0.1	6.0	3.2	—	>32	2.6	2.2	6.0	2.0
0	0.3	—	6.0	5.2	20	—	2.5	2.1	3.5	0.5
0	0.1	0.1	5.2	9.4	52	94	2.3	1.4	3.5	—
0		—		7.5		—		1.9		3.1
Hydrocortisone (F)										
25		*0.4*		4.5		11		1.7		*7.8*
100	*0.7*	*0.9*	*7.2*	7.7	10	9	2.5	*3.2*	*8.9*	*3.5*
400		*6.8*		*13.3*		2		*5.4*		*7.8*
Cortisone (E)										
25	*0.8*	*0.7*	*8.7*	5.9	11	8	2.0	1.6	*8.9*	*5.3*
100	*1.2*	*1.2*	*16.2*	*16.3*	14	14	*3.5*	*2.8*	*6.6*	*9.1*
200‡	*5.9*‡		*20.9*‡		4‡		*2.8*‡		*10.4*‡	
11-Desoxy-17-hydroxy corticosterone (S)										
25		*0.9*		8.8		10		*3.3*		*3.8*
100	*2.3*	*1.0*	*23.3*	*10.0*	10	10	*2.7*	2.2	*10.9*	2.5
		3.0		*18.9*		6		*2.8*		*7.6*
Corticosterone (B)										
90 (As alcohol)		*4.2*		*12.5*		3		*3.1*		2.5
11-Dehydrocorticosterone (A)										
100		*6.9*		*25.4*		4		*3.9*		*5.6*
200		*12.2*		*19.1*		2		*2.8*		*6.7*
11-Desoxycorticosterone (DOC)										
25	*0.9*	*0.4*	*8.8*	4.5	10	11	*3.3*	*2.4*	*6.9*	1.8
100	*1.5*	*1.8*	*15.2*	*15.1*	10	8	*2.7*	2.2	*7.3*	1.7
200		*5.6*		*30.6*		5		*3.0*		*5.3*
Intramuscular injection										
Cortisone (E)										
25	*0.6*		*8.8*		15		2.5		4.2	
200		*0.7*		5.8		8		*2.8*		*7.5*
11-Desoxycorticosterone (DOC)										
25	*1.1*		*9.2*		8		*3.1*		*9.1*	

* Italicized numbers indicate values greater than the highest control value for the subject. Subject JO. was a man, AL. a woman, both with severe adrenal cortical insufficiency.

† AKSM = Alpha-ketolic steroid metabolites.
G'ase R-L = Lipid-soluble reducing substance extractable after glucuronidase and acid hydrolysis.
Acid R-L = R-L after acid hydrolysis only.
17-KS = Total neutral 17-ketosteroids.

‡ The 200 mg. dose of cortisone was administered to subject SCH., an orchidectomized, adrenalectomized man.

drocortisone (18) establishes the fact that 11-oxygenated steroids with a keto group on carbon atom 17 may be formed from 17-hydroxylated alpha-ketolic steroids. In addition, etiocholane-3α-ol-17-one has been identified following the administration of compound S (26).

Thorn and his co-workers in their review (25) state that corticosterone, although increasing "17-ketosteroids," is by no means as potent in this respect as F or E. In our single experiment, there was no increase in "17-ketosteroids" when 90 mg. of B alcohol was administered. However, we have found that levels above those of the highest control values were present after administration of 100 and 200 mg. of A_{ac} and 200 mg. of DOC_{ac}. The identification of neutral 17-ketosteroids following administration of alpha-ketolic steroids without a hydroxyl group at carbon atom 17 has not been reported (4). It seems probable that the increases noted with these alpha-ketols without a 17-hydroxyl group were primarily due to keto groups at other than the 17-position since the Zimmerman reaction is not specific for 17-ketosteroids.

DISCUSSION

The terminology has been defined and the limitations of the methods discussed in the section on Methods. The exact meaning of the word "identification" as used in this paper has been defined in terms of the methods used (see Methods and Table I). Comments have been made after each section in Results. Therefore, only the more general implications will be discussed here.

The most prominent urinary alpha-ketolic metabolites of the physiologically active compounds (F, E, S, B, A, and DOC) administered to individuals with little or no adrenocortical function have been found to be the 3α-hydroxy pregnane ("tetrahydro H_4") derivatives of the individual parent compound. The finding of the 3α-hydroxy normal configuration is in accord with previously reported work with compounds F (18) and E (19, 21). It is considered likely that the so-called "dihydro" (H_2) forms may, at least in part, be intermediates in the reduction of ring A to the tetrahydro form since Schneider (27) has identified "dihydro-E" (H_2E) in normal male urine. In addition, Savard and Goldfaden (28) have recently demonstrated the production of H_4F from H_2F, and Ungar, Davis, Rosenkrantz, and Dorfman (29) H_4S from H_2S. In addition, we have found small amounts of a substance that is probably H_2E (see Table I, exp. No. 3, peak Y_1) after administration of 200 mg. of E acetate per 24 hours to an adrenalectomized man.

These findings, in general, lend support to the tentative generalization of Dorfman and Ungar (4) that "the presence of the side chain at carbon-17 directs the reduction of the double bond of the Δ^4-3-ketone in ring A primarily to the 5-β configuration whether or not an oxygen function is present at carbon-11." However, the recent work of Engel, Carter, and Springer (30) and findings of our own suggest that corticosterone (B) and possibly 11-dehydrocorticosterone (A) more readily form the allopregnane derivative than those alpha-ketolic steroids having a 17-α-hydroxy group (E, F, and S). Engel and his co-workers have isolated and identified relatively large amounts of allopregnane-3α, 11β, 21-triol-20-one (allo-H_4B) from the urine of a man with rheumatoid arthritis given corticosterone. Our findings bearing on this problem are as follows: The Y_2 peaks after administration of compound A (exp. No. 5) and compound B (exp. No. 12) have similar characteristics as indicated in Table I. Similar characteristics including the absorption spectrum in sulfuric acid have been found for allopregnane-3α, 11β, 21-triol-20-one (allo-H_4B) isolated from the urine of a woman receiving ACTH (31), and identified by infrared spectrometry through the courtesy of Dr. Thomas F. Gallagher. An authentic sample synthesized by Dr. A. L. Kemp of the Sloan-Kettering Institute for Cancer Research, was used as the standard. It, therefore, seems probable to us that the Y_2 peaks in experiments No. 5 and No. 12 were in fact the 3α-hydroxy allo form of H_4B. Less complete evidence suggests that the Z_2 peak in these two experiments is an isomer of H_4A probably the 3α-hydroxy allo form. The R_{DOC} of the free and acetylated peak Z_2 suggests that it may be a tetrahydro derivative of A having either the allo 3α or the normal 3β configuration. The similarity of the sulfuric acid absorption spectra to that of H_4A also indicates that this compound is a H_4 derivative of compound A. Unfortunately, insufficient material was available for adequate infrared spec-

trometry because of loss or alteration in the final purification procedures. Assuming these peaks to be in fact the 3α-hydroxy allo derivatives, the ratio of the pregnane derivatives of compounds A and B (Table II) to their allo isomers varied from 1.5 to 5.

In addition to the reduction in ring A, we have found, as have others, that compounds F (18) and E (19–21) are interconvertible and in addition have found compounds B and A to be interconvertible. The direct conversion products as well as their tetrahydro derivatives were found in the urine. It seems probable that interconversion of the hydroxyl and keto groups at carbon atom 11 may also occur after reduction of ring A of compounds B and A, since Savard and Goldfaden (28) have recently demonstrated that the 11β-hydroxyl group is interchangeable with the 11-keto function of tetrahydro F and dihydro F.

SUMMARY

1. Hydrocortisone (F), cortisone (E), 17-hydroxy-11-desoxycorticosterone (S), corticosterone (B), 11-dehydrocorticosterone (A), and 11-desoxycorticosterone (DOC) have been administered to subjects with adrenal cortical insufficiency, and the major urinary alpha-ketolic steroid metabolites identified and semiquantitative estimations made of excretion. Identification procedures included R_{DOC} in various solvent systems, special color reactions and infrared spectrometric analysis and spectrometry in sulfuric acid or both.

2. The tetrahydro (H_4) form (3α-hydroxy pregnane configuration) of each was identified as a major alpha-ketolic metabolite. However, corticosterone (B) and 11-dehydrocorticosterone (A) each give lesser amounts of two additional alpha-ketolic metabolites saturated in ring A which appear to be the 3α-hydroxy allopregnane derivatives of the parent compound and its C-11 interconversion product.

3. The interconversion of the keto and hydroxyl group at carbon atom 11 was demonstrated in the absence of a normally functioning adrenal cortex for corticosterone (B) and 11-dehydrocorticosterone (A) as well as for cortisone (E) and hydrocortisone (F) by the identification of the appropriate conversion products and their "tetrahydro" derivatives.

4. The general similarity of the chromatographic pattern after administration of F and E as well as the similarity after B and A was demonstrated.

5. The urinary excretion of total alpha-ketolic steroid metabolites (AKSM), reducing lipids extracted after glucuronidase plus acid hydrolysis (G'ase. R-L), and those extractable after acid hydrolysis alone (Acid R-L), and the total 17-ketosteroids (17-KS) was, in general, increased by the administration of these compounds. Considerable variability was experienced in these measurements.

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DISTURBANCES OF CYSTINE METABOLISM IN LIVER DISEASE

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The urinary excretion of cystine, as determined on the polarograph, varies, in normal adults, between 40 and 80 mg. daily (1). It may be increased either as a result of a low renal threshold for cystine or a raised plasma cystine concentration. Cystinuria due to a low renal threshold occurs in 'classical cystinuria,' the Fanconi syndrome and hepatolenticular degeneration (2). In such conditions the excretion of cystine is usually large, 0.5 to 1.0 g. daily, and is associated with other characteristic changes in the pattern of amino acids excreted. A moderately increased excretion of cystine has been described in patients with liver disease (3–5) and this has been shown, by paper chromatography, to result from a raised plasma cystine concentration. It has been suggested that the increased urinary cystine in these patients might form a sensitive index of impaired amino acid metabolism (6).

Using the quantitatively more accurate method of polarography for cystine assay we have reinvestigated the plasma level and urinary output of cystine in cases of liver disease. In addition we have carried out cystine and cysteine tolerance tests comparing the results found in patients with chronic liver injury with those found in normal subjects.

METHODS

Polarography (7) was used to measure the concentration of cystine in plasma and the output of cystine in urine. Technical details have already been published (8). This method does not distinguish between cystine and cysteine as the S-S linkage is reduced to the S-H form in the polarizing cell. It is improbable that cysteine, following its ingestion, would remain for long in the reduced state in the plasma as the oxygen present, at normal plasma pH, would rapidly cause its conversion to cystine. This problem has been discussed in more detail elsewhere (8). Recoveries of cystine added to plasma were accurate to ± 10 per cent. Comparing our results with those of Stein, Bearn, and Moore (9), who used the method of elution chromatography, we find that the normal plasma cystine levels are very similar; their figures for the 24-hour excretion of cystine in normal human urine, however, are consistently lower (9, 10) than those that we have found or were found by Reed (1). Measurable quantities of glutathione are absent from normal human plasma and urine. Even if, under the present experimental conditions, it should appear, the polarographic method as used, with the cobaltous ion as catalyst, does not determine glutathione (11). Possibly some cystine containing peptides may produce a catalytic polarographic wave at the same voltage as cystine itself. Unfortunately there is no feasible way to check this directly with the polarograph. Paper chromatographic studies have been made of both plasma and urine of normal and cystinuric subjects after feeding either cystine or cysteine. These showed changes in the cystine concentration roughly paralleling the polarographic analysis after similar feeding experiments done, in some cases, on the same subjects (8, 12). Likewise some of the experiments described here on patients with liver disease have also been checked by paper chromatographic analysis. No evidence has been obtained for the presence of cystine containing peptides in plasma or urine. In view of the slight doubt that our plasma cystine levels are due to cystine or to a combination of cystine with cysteine or, in the case of urine, partly to an unidentified complex of cystine we will refer to the substance throughout as 'cystine.'

Observations were made on normal subjects and on patients with both acute and chronic liver disease. The important clinical features and biochemical findings of the latter are given in Table I. The 24-hour excretion of 'cystine' in the urine was estimated from 19 patients with cirrhosis on 90 occasions, from 10 patients with acute hepatitis on 16 occasions and from one patient with acute hepatic necrosis on 8 occasions. Forty-six determinations were made of the fasting plasma 'cystine' level on 18 patients with cirrhosis. The control observations consisted of 25 determinations of the 24-hour output of 'cystine' on 25 normal adults and 50 fasting plasma 'cystine' levels on a further 16 normal subjects, these latter have also been described elsewhere (8). All patients were on the usual ward diet and none were receiving protein supplements.

Experiments were carried out in which l-cystine, 5.0 g., was given by mouth to fasting patients; the plasma 'cystine' level was estimated immediately before and at

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TABLE I

Summary of clinical and biochemical findings in patients with hepatic disease

Patient	Sex	Age (yrs.)	Clinical diagnosis	Histology of liver	Esophageal varices	Serum bilirubin mg./100 ml.	Serum proteins g./100 ml.		Flocculation tests		Fasting plasma cystine, μg./ml. (highest reading)†
							Albumin	Globulin	Cephalin cholesterol	Thymol turbidity (units)	
R. C.	M	56	Alcoholic cirrhosis	Advanced active multilobular cirrhosis	Present in lower esophagus	5.0	1.3	4.7	3+	18	20.2
K. M.	M	51	Portal hypertension Portal vein thrombosis	Normal liver	Very extensive	0.3	4.3	2.9	2+	3	12.6
D. A.	M	47	Post hepatitis Fibrosis	No biopsy because of hypoprothrombinemia	Small	1.0	2.9	1.8	3+	6	17.8
F. B.	M	53	Hepatic cirrhosis Volvulus of stomach	Advanced active multilobular cirrhosis	No	0.4	3.7	2.9	+	12	9.1
M. C.	M	68	Atrophic alcoholic cirrhosis	Diffuse fibrosis, severe liver cell damage	No	12.0	3.0	3.7	2+	12	20.4
A. G.	F	68	Obstructive jaundice Biliary cirrhosis	Advanced biliary cirrhosis, bile lakes	Small (*post-mortem* finding)	10.0	2.7	3.1	+	7	43.2
C. G.	F	48	Chronic hepatitis	Chronic hepatitis with marked cellularity of fibrous tracts	Small	2.1	3.3	4.9	2+	18	12.6
I. M.	F	55	'Primary biliary cirrhosis'	Multilobular fibrosis Bile duct regeneration	Prominent abdominal veins, no varices	4.9	4.2	3.5	+	6	11.7
M. W.	F	47	Syphilitic hepatic fibrosis	No biopsy because of hypoprothrombinemia	No	2.0	2.8	2.2	+	3	12.6
A. R.	M	37	Hepatic cirrhosis	No biopsy	1) Varices present	0.4	3.3	2.9	2+	7	8.4
					2) Lieno-renal shunt	0.6	3.1	4.2	2+	5	8.2
G. M.	F	52	Hepatic cirrhosis	Portal cirrhosis with marked cellularity of portal tracts	Very extensive varices and lieno-renal shunt	0.8	3.5	4.3	2+	7	11.7
G. A.	M	23	Infective hepatitis	No biopsy	No	1.0	4.1	3.6	+	10	12.6
J. B.	M	28	Infective hepatitis	No biopsy	No	17.8	4.2	3.0	-ve	1	13.1
I. T.	M	58	Serum hepatitis	Periportal inflammation and fatty change*	No	3.2	4.2	2.2	-ve	3	11.1

* Photomicrograph of this biopsy published elsewhere (6), Plate 36, Figure 12.
† Normal plasma cystine = 10.64 μg./ml.

one-half, one, two, four, and six hours afterwards. The same measurements were also made after the ingestion of 3.0 g. of l-cysteine HCl. For control observations a similar series of tests were carried out on normal subjects. The smaller dose of cysteine HCl was chosen as this amino acid has a tendency to produce nausea and vomiting, which we wished to avoid in patients with liver injury, and because, in experimental animals, single large doses may give rise to toxic side effects (13).

RESULTS

1) *The fasting plasma 'cystine' concentration*

The normal figures varied between 6.7 and 14.4 μg. per ml. with a mean of 10.64 ± 1.87. In patients with hepatic cirrhosis the fasting plasma 'cystine' level varied between 5.6 and 43.2 μg. per ml. with a mean of 13.7 ± 5.75. In general it was found that in patients with cirrhosis the higher plasma 'cystine' levels were associated with the more severe degrees of liver damage: The patient with a 'cystine' concentration of 43.2 μg. per ml. was in fact in the terminal stage of hepatic coma at the time. These results are expressed as a histogram, Figure 1.

Sufficient observations on patients with acute hepatitis have not been made to allow of any conclusions, but in one case of massive hepatic necrosis the plasma 'cystine' reached a concentration of 70 μg. per ml. shortly before death.

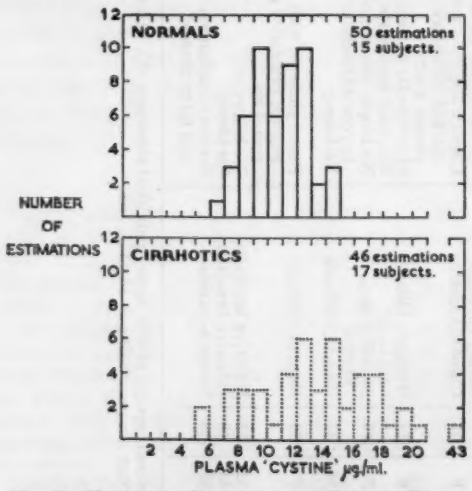

FIG. 1. HISTOGRAM COMPARING THE FASTING PLASMA CYSTINE LEVEL IN NORMAL SUBJECTS WITH A SIMILAR SERIES OF RESULTS FROM PATIENTS WITH HEPATIC CIRRHOSIS

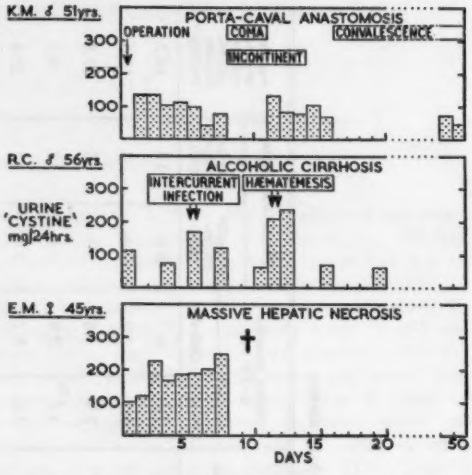

FIG. 2. DAILY CYSTINE EXCRETION IN THREE PATIENTS; TWO, K. M. AND R. C., WITH CHRONIC LIVER DAMAGE AND ONE, E. M., WITH MASSIVE HEPATIC NECROSIS

This shows the variations which can occur in the urinary cystine corresponding approximately with the clinical condition of the patient.

2) *The excretion of 'cystine' in the urine*

In 13 adult normal males the output of 'cystine' varied between 43 and 70 mg. per 24 hours, with a mean of 61 mg. In 12 adult normal females the range was between 28 and 53 mg. of 'cystine' with a mean of 44 mg. per 24 hours. In patients with cirrhosis the 24-hour excretion of 'cystine' was measured on 90 separate occasions in 19 different patients (10 male, 9 female), the majority being represented by more than one urine specimen. As the output of 'cystine' was not constant for any given patient but fluctuated with the severity of the illness (Figure 2) each estimation is treated as a separate entity on the histogram (Figure 3). The 10 male patients provided 59 urine specimens, the 'cystine' excretion varied between 38 and 234 mg. per 24 hours with a mean of 81 mg. From the 9 female patients 31 urine specimens were obtained, the range of 'cystine' excretion was from 32 to 150 mg. with a mean of 65 mg. per 24 hours. The mean for each group shows an increase in 'cystine' excretion of 20 mg. per 24 hours when compared with the corresponding average normal figure. This difference, though appreciable, does not result in a sharp separation between normal

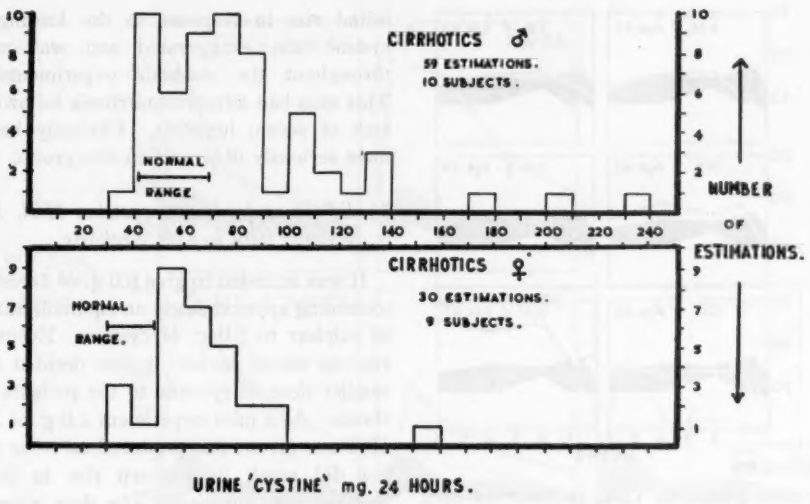

FIG. 3. HISTOGRAM SHOWING THE RANGE OF VARIATION IN THE 24-HOUR EXCRETION OF CYSTINE IN MALE AND FEMALE PATIENTS WITH HEPATIC CIRRHOSIS

subjects and patients with cirrhosis since the 'cystine' excretion in approximately half the estimations on the latter fall within the normal range. There appeared to be a definite correlation between the 'cystine' excretion and the severity of the liver injury; the highest figures for urinary cystine excretion being found in those patients with the most severe illness.

In 10 patients with acute hepatitis the 'cystine' excretion during the acute phase of the illness was elevated, averaging 97 mg. and varying between 76 and 134 mg. per 24 hours; this returned rapidly to within normal limits during convalescence, the mean figure at this stage being 56 mg. and the range between 42 and 68 mg. per 24 hours. In one patient with massive hepatic necrosis the excretion of 'cystine' rose to 250 mg. per 24 hours two days before death.

TABLE II

Plasma cystine concentration after feeding 5.0 g. of cystine

Patient	Plasma cystine concentration, μg./ml.					
	Fasting	½ Hour	1 Hour	2 Hours	4 Hours	6 Hours
(a) To patients with hepatic cirrhosis						
K. M.	12.6	10.0	10.8	16.1	15.7	13.0
F. B.	9.1	12.6	13.1	16.2	15.7	12.2
M. W.	9.0	12.7	18.6	16.3	19.3	15.3
A. G.	7.2	9.0	14.0	17.5	13.1	10.8
C. G.	8.0	8.2	12.7	13.5	13.0	15.5
D. A.	17.8	15.8	16.2	25.0	23.0	25.6
(b) To patients with acute hepatitis						
I. T.	7.5	7.5	15.5	13.8	—	10.1
J. B.	13.1	12.1	15.2	14.5	14.5	9.0
G. A.	12.6	13.1	12.8	17.1	16.2	—
Average normal	10.8	12.8	13.3	13.3	12.7	11.3
Maximum normal	12.6	16.9	16.2	14.5	15.2	13.2
Minimum normal	7.8	9.6	9.0	12.6	9.0	9.0

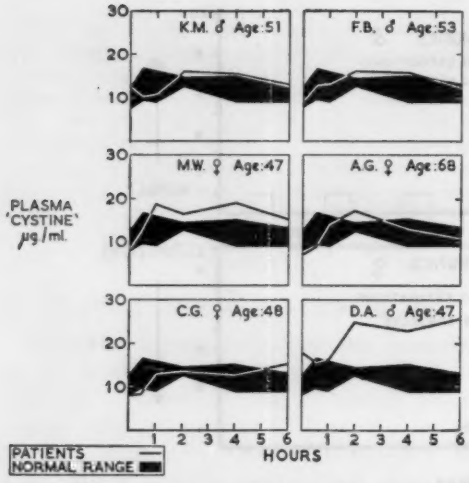

FIG. 4. CYSTINE TOLERANCE TESTS IN PATIENTS WITH HEPATIC CIRRHOSIS

Cystine 5.0 g. was given to each patient immediately after the fasting blood was drawn. The control results show the extreme range of normal variation in seven similar studies on normal subjects.

3) *Effect of feeding 1-cystine, 5.0 g., to patients with hepatic cirrhosis*

The results are given in Table II and Figure 4; the control observations were obtained from seven similar studies on normal subjects and are published in detail elsewhere (8). In five of the six patients the cystine tolerance curve deviated little from that found in the controls although the peak was somewhat delayed. In the case of D.A. the fasting plasma 'cystine' level was elevated and the initial rise in response to the loading dose of cystine was exaggerated and was maintained throughout the six-hour experimental period. This man had advanced cirrhosis following an attack of serum hepatitis. Clinically he was the most seriously ill patient in this group.

4) *Effect of feeding 1-cysteine HCl, 3.0 g., to patients with hepatic cirrhosis*

It was intended to give 6.0 g. of 1-cysteine HCl containing approximately an equimolecular amount of sulphur to 5.0 g. of cystine. Eventually, for reasons stated earlier, it was decided to give a smaller dose of cysteine to the patients with cirrhosis. As a pilot experiment 2.0 g. of 1-cysteine HCl was given; this produced no toxic symptoms but did result in a sharp rise in the plasma 'cystine' concentration. The dose given in subsequent tests, and also to the control subjects, was 3.0 g. The average figure for the four normal subjects shows that the plasma 'cystine' rose from a fasting level of 9.0 μg. per ml. to 15.3 μg. per ml. at one hour but returned to the fasting level by the end of two hours. In the patients with cirrhosis the rise was very much greater. In the case of M. C. the peak 'cystine' concentration, reached at one hour, was 60 μg. per ml. and it exceeded 30 μg. per ml. in all of the cases investigated. The results are given in detail in Table III and also in Figure 5.

Of the five patients with cirrhosis fed cysteine two (G. M. and A. R.) had lieno-renal anastomoses which had been constructed for the relief of portal

TABLE III

Plasma 'cystine' concentration after feeding 3.0 g. of 1-Cysteine HCl

Patient	Plasma 'cystine' concentration, μg./ml.					
	Fasting	½ Hour	1 Hour	2 Hours	4 Hours	6 Hours
	(a) To patients with hepatic cirrhosis					
C. G.	12.6	47.6	42.5	23.4	16.2	14.2
I. M.	11.7	56.7	49.3	46.5	25.6	18.6
M. C.	16.7	52.2	60.0	42.2	27.2	22.4
G. M.	11.7	17.5	37.5	28.1	19.5	15.6
A. R.	8.2	33.3	42.5	27.1	27.8	17.8
	(b) To controls					
M. S.	9.4	12.4	19.9	12.1	8.7	10.3
J. C.	6.5	9.2	12.9	9.1	9.9	9.5
E. S.	11.1	19.7	11.3	12.5	14.1	8.4
T. G.	9.3	10.9	17.0	10.8	8.7	8.3

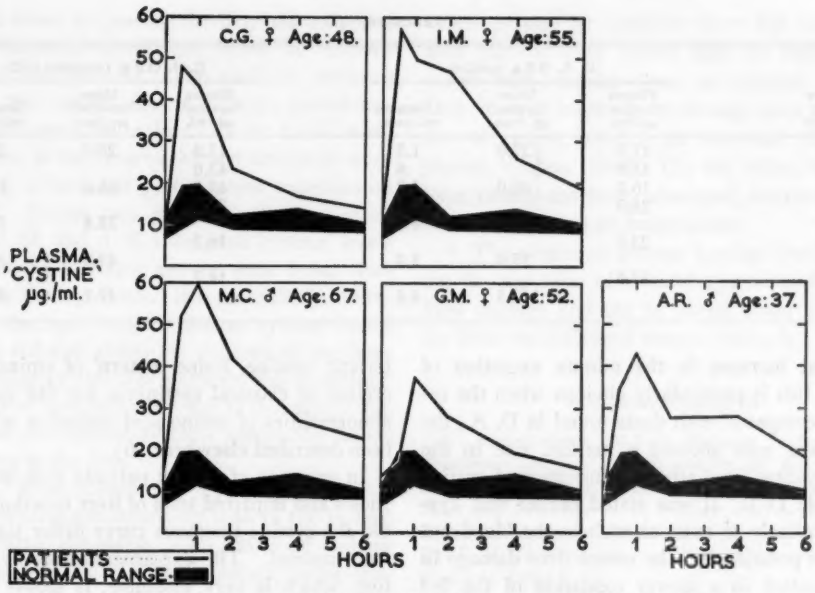

FIG. 5. CYSTEINE TOLERANCE TESTS IN FIVE PATIENTS WITH HEPATIC CIRRHOSIS
1-Cysteine HCl 3.0 g. was given immediately after the fasting blood was drawn. The control results show the extreme range of variation on four normal subjects.

hypertension. The results in these two cases did not differ materially from those found in the three patients with cirrhosis but no clinical evidence of portal hypertension. The presence of these large portal-systemic anastomotic veins did not lead to an increased abnormality in the shape of the cysteine tolerance curves.

5) *'Cystine' clearance in patients with liver injury*

In normal subjects the urine excretion of 'cystine' varies directly with the plasma concentration (8). In certain of the cystine and cysteine tolerance tests carried out on patients with cirrhosis urine samples were collected during the experimental period and it was found that the 'cystine' clearance was the same in these patients as in the controls, that is around 2 to 4 ml. per min. at all plasma concentrations investigated. These results have been expressed graphically (Figure 6), plasma' cystine' concentration being plotted against the minute excretion of 'cystine.' The figures show a highly significant correlation between the plasma level and the urine excretion of 'cystine,' the correlation coefficient is $r = 0.8020$.

The figures in this graph do not include those from the cysteine tolerance test on C. G. in which urine 'cystine' was estimated. In this experiment at the highest plasma 'cystine' levels there was no

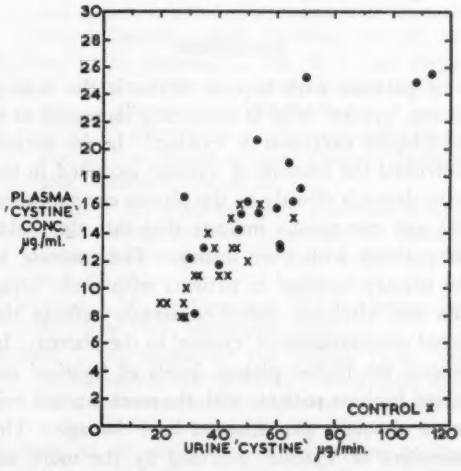

FIG. 6. GRAPH SHOWING THE RELATIONSHIP BETWEEN THE PLASMA 'CYSTINE' CONCENTRATION AND THE MINUTE EXCRETION OF 'CYSTINE' IN THE URINE (THE CORRELATION COEFFICIENT IS $r = 0.80$)
● = Patients with hepatic cirrhosis.
× = Normal controls.

TABLE IV

Time in hours	D. A. (5.0 g. cystine) Plasma 'cystine' μg./ml.	Urine 'cystine' μg./min.	Clearance ml./min.	C. G. (3.0 g. l-cysteine HCl) Plasma 'cystine' μg./ml.	Urine 'cystine' μg./min.	Clearance ml./min.
0	17.8	27.0	1.5	12.6	28.8	2.3
½	15.8			47.6		
1	16.2	60.0	3.8	42.5	66.6	1.4
2	25.0			23.4		
3		108.0	4.3		78.8	3.4
4	23.0			16.2		
5		55.0	2.4		49.8	3.1
6	25.6			14.2		
7		112.5	4.4		47.2	3.3

very great increase in the minute excretion of 'cystine'; this is particularly obvious when the results are compared with those found in D. A., the only patient who showed a marked rise in the plasma 'cystine' level after the ingestion of cystine (see Table IV). It was stated earlier that cysteine is unlikely to exist as such in the blood but it remains possible that the severe liver damage in C. G. resulted in a slower oxidation of the fed cysteine. If, as is also possible, cysteine is reabsorbed more readily than cystine by the renal tubule this would account for the lower minute excretion of 'cystine' by C. G. at the very high blood levels achieved and also for the rising urine excretion co-incident with the falling blood level during the latter part of the test period.

DISCUSSION

In patients with hepatic cirrhosis the fasting plasma 'cystine' level is frequently increased as is the 24-hour excretion of 'cystine.' In the normal individual the amount of 'cystine' excreted in the urine depends directly on the plasma concentration (8) and our results indicate that this also holds for patients with liver injury. The increase in the urinary 'cystine' in patients with acute hepatitis and cirrhosis therefore simply reflects the raised concentration of 'cystine' in the plasma. In general the higher plasma levels of 'cystine' occurred in those patients with the most marked evidence of severe parenchymal liver damage. The quantities of 'cystine' excreted by the more severely ill patients were of the same order that Harris and Warren (14) found in heterozygous cystinuric subjects. However the urine of patients with liver disease did not show the characteristic 'cystine, lysine pattern' of amino acid excretion of classical cystinuria but did show other abnormalities of amino acid excretion which have been described elsewhere (6).

In only one of the six patients with hepatic cirrhosis and impaired tests of liver function (D. A.) did the cystine tolerance curve differ significantly from normal. The explanation may be that cystine, which is very insoluble, is slowly absorbed from the gut and does not reach the liver in high concentration. It cannot act, therefore, as a significant load and a markedly abnormal tolerance curve is not produced except in the presence of very severe liver damage. Intravenous administration of cystine would be necessary to clarify this point but because of its extreme insolubility at body pH this is impracticable.

Cysteine, being very soluble, is rapidly absorbed and presumably reaches the liver in relatively high concentration either as cysteine or as cystine. Consequently 3.0 g. of l-cysteine HCl produced a greater percentage rise in the plasma 'cystine' level of normal subjects than did 5.0 g. of cystine. Other workers have reported results similar to these, on normal subjects, and have offered the same explanation (8, 12, 15). These authors pointed out that this was also the probable explanation of the results of the feeding experiments of Brand, Cahill, and Harris (16) who, however, analyzed only urine for cystine and interpreted their results differently. The patients with cirrhosis fed 3.0 g. of l-cysteine HCl all showed a rise in the plasma level markedly greater than that produced by the same dose in the control subjects. This phenomenon could be due either to a reduced ability on the part of the liver to metabolize cystine (or possibly only cysteine) or to

the portal blood by-passing the liver *via* collateral venous channels, which can often be demonstrated in such patients. The available evidence supports the former theory. Large portal-systemic anastomotic veins in C. G. or I. M. were not detected at the time of the test and none were found at a subsequent *post mortem* examination on M. C. In the two patients with lieno-renal shunts (G. M. and A. R.) the peak 'cystine' levels in the cysteine tolerance curves were lower than in the other three patients. It is more likely, therefore, that the high levels of plasma 'cystine' found indicate a reduced ability on the part of the liver to metabolize cystine (or possibly only cysteine) at the normal rate, probably due to damage to one or more of the enzyme systems specifically handling cystine in the liver.

In these experiments only 'cystine' metabolism has been studied quantitatively both in the fasting state and after the stress of an oral load and no conclusions can be drawn therefore, from this work, about the metabolism of other amino acids. The possibility remains that in the course of progressive hepatic disease, as different enzyme systems are affected, so the ability of the liver to utilize individual amino acids becomes impaired. Chromatographic studies reported elsewhere also support this theory (6). Cystine however appears to be one of the first amino acids to show disturbed metabolism in liver disease.

SUMMARY

1. The plasma 'cystine' concentration and urinary 'cystine' excretion have been measured in patients with acute and chronic liver disease, using a polarographic method of determination.

2. Comparison with the normal shows that: 1) The average figure for the plasma 'cystine' level is higher for patients with hepatic cirrhosis and shows a wide range of variation. 2) The average output of 'cystine' in the urine per 24 hours is greater in the patients with liver disease. 3) The increased output of 'cystine' is directly due to the raised plasma concentration and not to any alteration in the renal clearance. The higher figures for both plasma and urine are found in those patients with the more severe liver injury.

3. Tolerance tests have been carried out in which cystine or cysteine were fed to patients with chronic liver injury and the results compared with similar tests on normal subjects. Only in very severe liver damage does the ingestion of cystine produce an abnormal rise in the plasma 'cystine' level. On the other hand cysteine caused markedly abnormal tolerance curves in all the patients so investigated.

4. The abnormal plasma 'cystine' levels following the ingestion of cysteine appear to be due to liver damage and not to portal blood by-passing the liver *via* collateral venous channels.

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STUDIES OF BACTERIAL TRANSFUSION REACTIONS FROM REFRIGERATED BLOOD: THE PROPERTIES OF COLD-GROWING BACTERIA [1]

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Transfusions of blood heavily contaminated with gram negative bacilli have led to violent reactions characterized usually by irreversible hypotension and death (1–4). These reactions have resulted not only from transfusion of blood contaminated with such bacteria as the coliforms, which grow best at 37° C., but also of blood contaminated with certain unusual gram negative bacilli which are capable of better growth in the refrigerator at 4 to 8° C. than in the incubator at 37° C. Such cold-growing bacteria have long been of interest to bacteriologists dealing with problems in the fish and dairy industries, but have only recently presented an important medical problem upon being identified as contaminants in transfused human blood. Their medical importance has been emphasized by Pittman (5), Stevens, Legg, Henry, Dille, Kirby, and Finch (4), and Wetterlow, Kay, and Edsall (6).

A study of warm-growing contaminants of transfused blood has recently disclosed that 2.27 per cent of bottles in the blood bank at the University of Michigan contained bacteria which could be cultured at 37° C. (7). Reactions from these bloods, contaminated by bacteria isolated at 37° C., occurred only rarely, however, because the degree of contamination was not great enough to initiate growth in the refrigerator during storage, or at room temperature during transfusion. The bactericidal or suppressive actions of the bloods themselves were found to be important in preventing growth of contaminants at both refrigerator and room temperatures. The factors which retarded the development of these warm-growing bacteria in blood protected the recipient not only from the direct effects of the bacteria, but also from the hemolysis, increased fragility, and rare change in type, which were observed during experimental contamination of banked blood.

Because cold-growing organisms also have been held responsible for bacterial transfusion reactions, the present study was undertaken to determine their potential menace as contaminants of transfused blood. When it was found that bacteria which grow well at 4 to 8° C. could be recovered in large numbers from likely sources of contamination in the blood bank, the following points were investigated as a means of evaluating their threat to recipients of refrigerated blood:

1) The rate of growth and survival of these bacteria in refrigerated blood and in blood at room temperature.

2) The effects of their growth on the blood itself.

3) The *in vivo* toxicity of these bacteria, as determined by experimental transfusions of animals.

4) Their susceptibility to agents which might be used to suppress their growth at low temperatures.

5) Protection of animals from their toxic effects.

EXPERIMENTAL METHODS

A. *Isolation of cold-growing bacteria from sources of contamination in the blood bank and vicinity*

Three methods were used to collect bacteria which could grow at 4 to 8° C.:

1) Samples of feces, nasal discharges, air, dust, ice, soil, and snow were inoculated directly onto tryptose agar slants and incubated in the refrigerator at 4 to 8° C.

2) Materials were obtained from persons and articles in the blood bank and inoculated into banked citrated blood and tryptose phosphate broth for incubation at 4 to 8° C. Minute samples of feces, dust, nasal secretions, air, and soil were used for this purpose in an attempt to reproduce roughly the conditions under which blood might be contaminated in the blood bank.

The inoculated bloods and broths were incubated at

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4 to 8° C. and observed for growth for three weeks. Growth in blood was determined by making frequent pour plates which were incubated at both 37° C. and 4 to 8° C.

3) Secreta, excreta, and exudates from patients and hospital personnel were cultured on blood agar at 37° C. and the bacteria isolated were identified and tested for growth at 4 to 8° C. by subculturing to tryptose phosphate broth and incubating in the refrigerator. In addition to these bacteria of human origin, bacteria were also isolated at 37° C. from articles in the donor room of the blood bank and elsewhere in the hospital for study of growth in the cold.

B. *Identification of isolated bacteria*

Among the bacteria isolated at 4 to 8° C., many were coliforms which gave typical reactions, and a very few were diphtheroids and staphylococci. The great majority, however, required special means of identification. Studies on routine fermentation were conducted with the basal medium of Ayers, Rupp, and Johnson as modified by Smith, Gordon, and Clark (8).

All strains were tested for pigment production at 4°, 27°, and 37° C. on 5 per cent glycerol agar, in asparagin solution, and on tryptose agar slants containing 0.1 per cent glucose as recommended by Pittman (5). Leifson's method (9) was used for staining of flagella on all bacteria isolated in the cold.

As a means of distinguishing *Pseudomonas aeruginosa* from other pseudomonads, growth was examined at 41° C. Ability to grow at 41° C. was considered a characteristic of *P. aeruginosa* only. All bacteria isolated in the cold were incubated at 27° and 4° C. on Simmon's citrate agar, rabbit blood agar, and EMB medium.

C. *The rate of growth and survival of bacteria at cold and warm temperatures in broth and blood*

The bacteria isolated at 4 to 8° C. were compared with those isolated at 37° C. for their respective abilities to grow in the cold and at warm temperatures. A total of 102 cold-isolated bacterial strains and 65 warm-isolated strains were each inoculated into 12 ml. of recently drawn citrated banked human blood so that the concentration would be 10^3 to 10^5 viable bacteria per ml. Each strain was also inoculated simultaneously into 12.0 ml. of tryptose phosphate broth. Each sample of blood was divided into two equal parts; one sample was incubated at room temperature (27° C.) and one in the refrigerator at 4 to 8°. Growth or death in blood was measured by frequent plate counts at 27° C. Each sample of broth was divided into three equal parts; then one part was incubated at 37° C., one at 27° C., and one at 4 to 8° C. Growth in broth during 21 days was measured turbidimetrically in a Coleman colorimeter.

A second group of bloods and broth were similarly inoculated, but with heavier suspensions of 68 other bacterial strains isolated at 37° C., so that the final concentrations ranged from 10^5 to 10^8 bacteria per ml.

D. *Determination of the susceptibility of cold-growing bacteria to antibiotics in broth.*

The observation that many strains of bacterial contaminants were able to grow well in refrigerated blood led to a search for antibiotics which could inhibit their multiplication at 4 to 8° C. Antibiotics listed in Table IV were tested against large inoculums of 10^8 to 10^9 bacteria per ml. in tryptose phosphate broth; their concentrations were 2 or 20 micrograms per ml. To achieve these concentrations in banked blood, it would be necessary to add only 1 or 10 mg., respectively, to each pint of blood. These broths inoculated with bacteria listed in Table IV were divided into two samples so that one could be incubated at 4 to 8° C. and one at 37° C., unless the bacteria were incapable of growth at 37° C. Bacteria unable to produce visible growth at 37° C. were incubated at 27° C. and 4 to 8° C. At the end of 24 hours, tubes incubated at 27° C. or 37° C. were examined for growth. Tubes incubated at 4° C. were examined periodically and results were recorded when control tubes, containing no antibiotics, had reached a turbidity equal to that of control tubes incubated for 24 hours at 27° C. or 37° C., as measured in the Coleman colorimeter.

E. *Determination of the susceptibility of cold-growing bacteria to antibiotics in human blood*

Because oxytetracycline, chlortetracycline, and polymyxin B inhibited in broth more strains at 4 to 8° C. than other antibiotics, they were also examined for their ability to suppress growth in blood in the refrigerator. Twenty ml. of human blood were inoculated with the bacteria listed in Table V to give bacterial counts of 10^3 to 10^5 per ml. A plate count was made immediately after inoculation and then 18 ml. of every sample of blood was divided equally among six tubes. Two of the inoculated tubes containing 20 micrograms per ml. of antibiotic and one without antibiotic were placed in the refrigerator and the corresponding three tubes were allowed to stand at room temperature. Frequent plate counts at 27° C. were made from the bloods containing antibiotics, as well as from the controls up to three weeks.

F. *Study of the toxicity of cold-growing bacteria*

The toxicity of living bacteria in rabbits: Two experiments were conducted. In the first experiment, serial dilutions of a saline suspension of bacteria grown in tryptose phosphate broth were added directly to rabbit blood immediately before transfusion. The purpose of this was to compare the approximate minimum lethal dose in transfused blood of cold-isolated bacteria with that of ordinary coliform bacteria isolated at 37° C. The 29 strains of bacteria listed in Table VI were used. Plate counts were made immediately after addition of bacteria and the inoculated bloods were then transfused back into the corresponding rabbits through the ear vein. Rectal temperatures were taken hourly, blood was drawn from the ear vein for bacterial counts, and necropsy was carried out immediately after death. Cultures were made

of blood from the heart and from sections of liver and spleen. Surviving rabbits were sacrificed in one week.

In the second experiment, blood inoculated with sublethal numbers (10^3 to 10^8) of a given strain of bacteria was stored in the refrigerator at 4 to 8° C. for 15 days and then transfused back into the rabbit from which it had been drawn. The purpose was to determine the amount of growth in refrigerated blood necessary to produce harmful effects or death. The following bacteria were used: four strains of *A. aerogenes*, three of *E. coli*, two of *P. aeruginosa*, one of *Pr. vulgaris*, one *Pseudomonas* sp. and five of *Achromobacter* sp.

The toxicity of crude endotoxin of cold-growing bacteria in rabbits and mice: The crude endotoxin consisted of a dried bacterial residue. After incubation at 27° C. for 72 hours, the bacilli had been washed with distilled water from nutrient agar which rested on one of the four surfaces of rectangular bottles of 500 ml. capacity. The bacteria were then washed with 95 per cent ethyl alcohol twice and acetone-ether once. The residue was brought to dry weight and stored in a dessicator with calcium chloride to prevent loss of potency due to absorption of water. Prior to use, a suspension was prepared in 5 per cent sterile, pyrogen-free dextrose solution containing 20 mg. per ml. of crude endotoxin. The toxicity in rabbits of two cold-isolated strains, Achromobacter 106A and Pseudomonad 29B, was compared with that of a strain of *E. coli* isolated at 37° C. Each crude endotoxin was injected intravenously into each of six rabbits in different doses ranging from 5 to 60 mg. per Kg.

The relative toxicity in mice of the crude endotoxin of cold-isolated and warm-isolated bacteria was determined by measuring the amount of crude endotoxin required to kill in 24 hours 50 per cent of inoculated animals. Six serial dilutions of crude endotoxin, ranging in dose from 10.0 mg. to .312 mg., were each inoculated intraperitoneally into 10 white mice, weighing about 20 grams each. Each dose was given in a volume of 0.5 ml. The LD_{50} was computed by the Reed-Muench formula (10) for the crude endotoxins prepared from the 30 bacterial strains shown in Table VII.

In order to check on the possibility that variation in bacterial toxicity resulted from dilution by non-bacterial products during preparation of crude endotoxin, the nitrogen content of the crude endotoxin was determined by the micro Kjeldahl method of Koch-McMeekin. In this way, the LD_{50} could be expressed as a function of total bacterial nitrogen, as well as of bacterial weight in milligrams.

G. *Protection against the toxic effects of gram negative bacilli*

Protection of recipient animals from contaminated transfused blood: On the basis of studies with antibiotics in section E, it was decided to test the effectiveness of oxytetracycline and chlortetracycline added prophylactically to stored blood in preventing the harmful manifestations of gram negative bacilli growing in the refrigerator.

Three strains of Achromobacter and three strains of Pseudomonas, which had been isolated in the cold, were inoculated into rabbit blood which was being "banked" at 4 to 8° C. In addition, three strains of bacteria, (coliform H., coliform 2Z and coliform BCX), which had been involved in two severe transfusion reactions in human beings, were similarly added to rabbit bloods. Each bacterial strain was added to three different samples of blood which had been drawn from the heart of three individual rabbits. Oxytetracycline was added to blood from one of the three rabbits, and chlortetracycline to blood from another to give a final concentration of 20 micrograms per ml. of blood. Thus two bloods contaminated with a given bacterial strain contained antibiotics. The 27 contaminated bloods were stored for 15 days in the refrigerator at 4 to 8° C. Upon removal from the refrigerator, plate counts of the bacteria were made and then the blood was transfused back into the rabbits from which they had been originally obtained. The clinical and pathologic studies described in section F were again performed and recorded in Table VIII.

Protection against killing by crude endotoxin: Both cortisone and the two antibiotics, chlortetracycline and oxytetracycline, were examined for their ability to save animals from lethal amounts of endotoxin. Rabbits received cortisone only, and mice received cortisone and antibiotics both singly and in combination. Fifty rabbits received intravenous injections of the crude endotoxin of either *E. coli* (G), Achromobacter 101A, or Pseudomonas 29B in amounts equivalent to approximately two M.L.D.'s each as calculated in section F. The dose of toxin per Kg. in each group of rabbits is recorded in Table IX. Each rabbit was injected intramuscularly with 15 mg./Kg. of cortisone at 24 hours and one hour in advance of the injection of endotoxin, unless otherwise stated in Table IX.

The doses of crude endotoxin and the doses and time of administration of cortisone or antibiotic are listed for mice in Table X. The antibiotics and cortisone were given intramuscularly to mice in volumes of 0.1 ml. and the crude endotoxin was injected intraperitoneally in 0.5 ml. of 5 per cent glucose.

RESULTS

A. *Isolation of cold-growing bacteria from sources of contamination in the blood bank and vicinity*

Material inoculated directly onto agar slants gave rise to frequent bacterial growth at 4 to 8° C., mainly from feces, dust, and soil. The bacteria grew as distinct colonies.

Blood and broth inoculated with feces was found to contain, on the average, 10^2 to 10^3 bacteria per ml. at the time of inoculation. The great majority (90 per cent) of bloods inoculated with feces manifested heavy growth at 4 to 8° C.

Sometimes this growth represented bacteria which reached a denser population in broth at 4° C. than 37° C. These were subsequently identified as achromobacters or as pseudomonads (see Section B). Often, however, coliform bacilli, which developed to a greater density in broth at 37° C. than at 4 to 8° C. were capable of growth in blood at 4 to 8° C. About one-third of the inoculated bloods in this group showed heavy growth in three days. In the remaining two-thirds, there was no growth until two to three weeks, at which time it became heavy. One factor responsible for early growth was a larger inoculum.

Bacteria isolated at 37° C. from the secreta, excreta, and exudates of patients, hospital personnel, and articles in the donor room of the blood bank were mainly coagulase negative staphylococci, *Escherichia coli, Aerobacter aerogenes, Proteus vulgaris, P. aeruginosa, Bacillus subtilis,* and diphtheroids. The ability of these bacteria to grow in the cold is described in Section C.

B. *Identification of bacteria isolated at 4 to 8° C.*

Except for the very rare isolation of a diphtheroid or staphylococcus (coagulase negative), all bacteria isolated at 4 to 8° C. were gram negative bacilli. Among 102 strains of these gram negative bacilli, which were subjected to detailed examination, 11 were identified as *Escherichia Freundii,* 2 as *E. coli,* and 8 as coliform bacteria of intermediate type. Of the remainder, 21 were classified as members of the genus *Pseudomonas* and 60 as members of the *Achromobacter* family. None of the pseudomonads were *P. aeruginosa.* Separation of the pseudomonads from the achromobacters is difficult and not satisfactory. Achromobacters and pseudomonads possessed in common the properties of good growth in the cold, poor metabolic activity, utilization of citrate on Simmons medium, non-fermentation of lactose, and fermentation of glucose to acid only. Separation of pseudomonads from achromobacter was based mainly on the production of pigment and the location of flagellae. If an organism produced green or yellow pigment and had lophotrichous flagellae, it was considered a member of the genus *Pseudomonas.* If no pigment was produced and if flagellae were peritrichous or absent, the strain was placed in the family *Achromobacter.* Undoubtedly, some of the organisms placed in the Achromobacter family could have been regarded as members of the genus *Alkaligenes.* In the present report, however, all bacteria isolated at 4 to 8° C. which fall into the *Achromobacter* family, will be referred to as *Achromobacter* regardless of the genus.

It is of interest that the pseudomonads isolated in the cold utilized asparagine more efficiently at 4 to 8° C. than at 27° C. or 37° C. for the production of pigment. A green pigment was readily produced by them in 10 to 14 days in the cold, but only a trace, or none at all, developed during their growth at the two warmer temperatures over the same period of time. Flagellae were produced equally well at all three temperatures but were retained longer in the cold.

C. *The rate of growth and survival of bacteria at cold and warm temperatures in broth and blood*

Growth of bacteria originally isolated in the refrigerator at 4 to 8° C. from human feces, earth, dust, and air: On the basis of turbidimetric growth in broth at three different temperatures, it has been possible to classify these cold-isolated gram negative bacteria into three groups regardless of species. The composition of these groups and their composite growth curves are presented in Figure 1. Growth curves in the three groups at 4 to 8° C. are quite similar to each other. The same is true of the growth curves at 27° C. The curves at 37° C. differ from group to group and are the main basis for the classification. Bacteria in Group I produced only slight, if any, turbidity at 37° C. There was good growth at 37° C. among bacteria in Group II, even though better growth occurred at 27° C.

There is a consistent difference, also, between the slope of the curves at 4 to 8° C. and at the two higher temperatures in all groups. At 4 to 8° C., it is difficult to discern the usual phases of growth: lag, logarithmic, and stationary. Instead, there is simply a slow but gradual increase in growth density during the entire incubation period of three weeks. There is a suggestion that the logarithmic phase is greatly prolonged at 4 to 8° C. and that if incubation were prolonged a stationary phase might ultimately be reached.

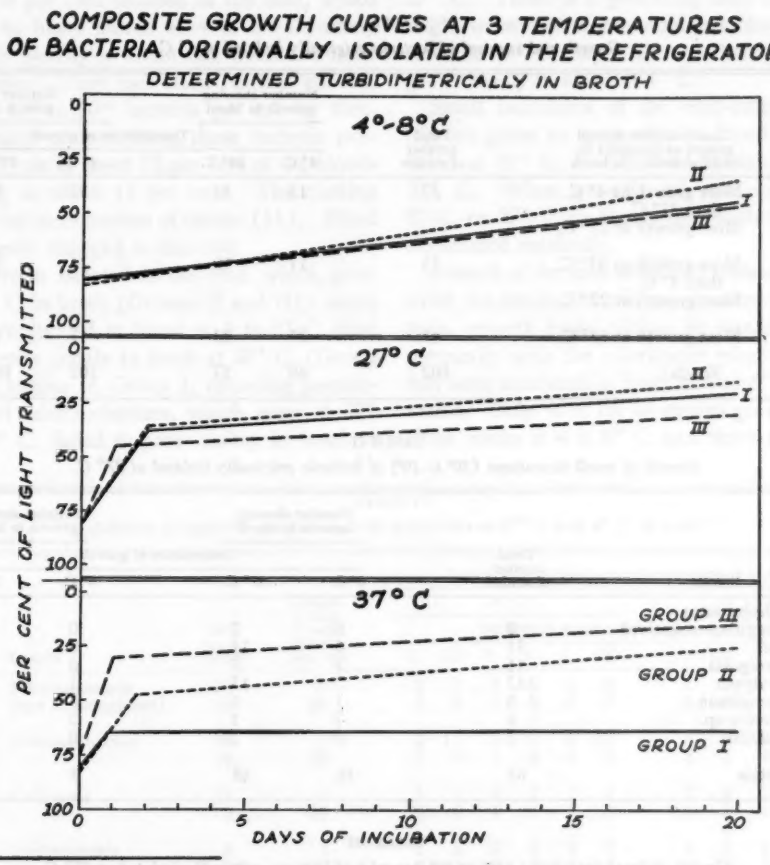

FIG. 1. CLASSIFICATION BASED ON FINAL TURBIDIMETRIC READINGS OF TOTAL GROWTH DENSITY

Group I—More growth at 4°–8° C. than 37° C. and most at 27° C.:
28 Achromobacters, 4 Pseudomonads.

Group II—Most growth at 27° C. More growth at 37° C. than 4°–8° C.:
11 Achromobacters, 10 Pseudomonads, 2 *E. Freundii.*

Group III—Most growth at 37° C.:
21 Achromobacters (mainly Alcaligenes), 21 Coliforms (11 *E. Freundii,* 2 *E. Coli,* 8 Intermediate Coliform), 5 Pseudomonads.

A composite curve is made by combining the individual growth curves for each of the bacterial strains in a group.

The three phases of growth are clearly defined when incubation in broth is carried out at 27° C.

On the basis of this classification, the growth and survival of these cold-isolated bacteria in blood and broth may be summarized as shown in Table I when small inoculums (10^2 and 10^3 viable cells per ml.) are used. The results may be compared to those summarized in Table II, which were obtained with small inoculums (10^2 and 10^3) of bacteria originally isolated at 37° C.; and with those summarized in Table III, which were obtained with large inoculums (10^5 to 10^8) of bacteria originally isolated at 37° C.

Among bacteria originally isolated in the cold,

TABLE I

Growth and survival of bacteria originally isolated at 4° C.

Group	Characteristics of total growth as measured by cultural density in broth	Total number of strains	Number showing growth in blood		Number showing growth in broth		
			Temperature of growth				
			4° C.	27° C.	4° C.	27° C.	37° C.
1	More growth at 4° C. than 37° C. Most growth at 27° C.	32	15	14	32	32	21
2	More growth at 37° C. than 4° C. Most growth at 27° C.	23	17	10	23	23	23
3	Most growth at 37° C.	47	37	33	47	47	47
	Totals	102	69	57	102	102	91

TABLE II

Growth of small inoculums (10^3 to 10^5) of bacteria originally isolated at 37° C.

Species	Total number of strains	Number showing growth in blood		Number showing growth in broth	
		Temperature of growth			
		4° C.	27° C.	4° C.	27° C.
Staphylococcus (coagulase-negative)	8	0	7	0	8
E. coli	21	6	11	0	21
A. aerogenes	11	3	8	0	11
Pr. vulgaris	13	3	13	1	13
P. aeruginosa	6	1	6	0	6
Paracolon sp.	4	3	1	2	4
B. subtilis	2	0	2	0	2
Totals	65	16	48	3	65

TABLE III

Growth of large inoculums (10^5 to 10^8 per ml.) of bacteria originally isolated at 37° C.

Species	Total number of bacterial strains	Number showing growth in blood			Number showing growth in broth		
		Temperature of growth					
		4° C.	27° C.	37° C.	4° C.	27° C.	37° C.
Staphylococcus (coagulase-negative)	22	6	20	20	10	22	22
E. coli	16	6	12	12	14	16	16
A. aerogenes	6	2	5	5	6	6	6
Pr. vulgaris	10	9	9	9	10	10	10
Paracolon sp.	5	4	5	5	5	5	5
P. aeruginosa	3	1	3	3	3	3	3
B. subtilis	4	2	2	2	Not tested	4	4
Diphtheroids	2	2	2	2	1	2	2
Totals	68	32	58	58	49	68	68

about 70 per cent grew in blood and 100 per cent grew in broth at 4 to 8° C. from small inoculums. These results are in contrast to those summarized in Table II which illustrate that growth in blood at 4 to 8° C., among small inoculums of bacteria isolated at 37° C. was not common (25 per cent) and growth in broth with these inoculums was very unusual (5 per cent).

Of the 70 per cent isolated in the cold, which grew well in blood stored at 4 to 8° C., many strains grew heavily in about one week and most by two weeks. They had often reached concentrations of 10^8 to 10^{10} bacteria per ml. by then. At refrigerator temperatures, these bacteria produced hemolysis in about 75 per cent of the bloods and clotting in about 15 per cent. The clotting was due to the consumption of citrate (11). Blood type was never changed in the cold.

Those strains isolated in the cold, which grew well at 37° C. in broth (Groups II and III), more frequently multiplied in blood at 4 to 8° C. than those that grew poorly in broth at 37° C. (Group I). Eight strains of Group I, including pseudomonads and achromobacters, which grew at 27° and 4 to 8° C., failed to grow visibly in broth at 37° C. There is a possibility that such bacteria might be missed if contaminated blood were cultured at 37° C. only. Two of these eight strains grew heavily in blood at 4° C.

Small inoculums of the cold-isolated bacteria did not grow as frequently in blood when incubated at 27° C. as did those bacteria isolated at 37° C. When growth did not occur at 4 to 8° C. or 27° C. in blood, the number of bacteria diminished markedly.

Growth of bacteria originally isolated at 37° C.: With the smaller inoculums of warm-isolated bacteria, growth could seldom be measured turbidimetrically with the colorimeter when the bacteria had been incubated in broth at 4 to 8° C. for three weeks. Only 16 of the 64 strains grew in blood in three weeks at 4 to 8° C. and never as heavily as

TABLE IV

Inhibition of bacterial contaminants by antibiotics at 37° C. and 4° C. in broth *

	Bacteria	Total no. of strains	Concentration of antibiotic *mcg./ml.*	Temperature of incubation during exposure to antibiotics: 4° C. — Number of strains whose growth was prevented: C	AU	P	T	S	AE	37° C. (or 27° C.) — Number of strains whose growth was prevented: C	AU	P	T	S	AE
	Pseudomonads	15	2	2	7	2	5	3	8	1	1	1	4	4	7
	(not *P. aeruginosa*)	11	20	2	8	2	8	6	7	6	6	1	7	9	8
	Achromobacter	28	2	2	11	1	3	4	12	5	6	1	5	7	13
		14	20	3	9	1	6	5	8	8	3	2	4	8	10
Isolated at 4° C.	Coliforms	11	2	1	2	0	2	2	3	0	1	1	1	0	1
		6	20	5	6	1	6	5	5	3	2	1	4	1	3
	Diphtheroids	4	2	2	3	2	2	0	2	3	2	3	3	2	3
		4	20	2	3	2	3	2	4	3	4	3	3	3	3
	Total of those	58	2	7	23	5	12	9	25	9	10	6	13	13	24
	isolated at 4° C.	35	20	12	26	6	23	18	24	20	15	7	18	21	24
	Proteus	6	2	2	1	0	3	2	3	0	1	0	0	3	2
		6	20	3	4	1	4	3	3	2	3	0	2	0	2
	E. coli	10	2	1	2	3	4	3	7	0	4	1	1	0	8
		10	20	3	4	5	5	3	6	10	7	2	7	7	7
Isolated at 37° C.	*A. aerogenes*	3	2	1	1	1	1	2	3	0	1	0	0	1	2
		3	20	2	2	1	1	2	3	3	3	0	3	3	3
	Coliforms	5	2	1	1	0	2	1	1	1	2	1	2	0	1
		5	20	1	1	0	3	2	2	4	3	1	4	2	2
	Total of those	24	2	5	5	4	10	8	14	1	8	2	3	4	13
	isolated at 37° C.	24	20	9	11	7	13	10	14	19	16	3	16	12	14
	Total of all strains	82	2	12	28	9	22	19	39	10	18	8	16	17	37
	capable of growth at 4° C.	59	20	21	37	13	36	28	38	39	31	10	34	33	38

* C = chloramphenicol; Au = chlortetracycline; P = penicillin; T = oxytetracycline; S = streptomycin; Ae = polymyxin B (aerosporin).

TABLE V

Inhibition of large bacterial inoculums in blood by tetracycline antibiotics in concentrations of 20 mcg./ml.

		Temp. (*degrees Centigrade*)	Total strains	Number of strains whose growth was prevented in blood		
				No antibiotics	Chlortetracycline	Oxytetracycline
Isolated at 4° C.	Achromobacter	4	26	8	25	25
		27	23	10	20	22
	Pseudomonads	4	17	2	16	17
		27	17	13	17	17
	Coliforms	4	16	0	11	13
		27	14	2	9	13
	Totals	4	59	10	52	55
		27	54	25	46	52
	E. coli	4	9	5	9	9
		27	10	5	10	10
	A. aerogenes	4	6	5	6	6
		27	6	2	6	5
Isolated at 37° C.	*Pr. vulgaris*	4	15	11	15	15
		27	5	0	5	5
	Intermediate Coliforms	4	2	0	2	2
		27	3	3	3	3
	Totals	4	32	21	32	32
		27	24	10	24	23
	Overall Totals	4	91	31	84	87
		27	78	35	70	75

cold-isolated bacilli. Nearly all strains of coagulase negative staphylococcus, *E. coli, Pr. vulgaris,* and *A. aerogenes* were killed in blood after three weeks in the refrigerator. Of the 16 organisms which grew at 4 to 8° C. in blood, 5 produced hemolysis. At 27° C., growth in blood was nearly as frequent with the smaller inoculums as with the larger.

With the larger inoculums of warm-isolated bacteria, growth in the refrigerator was about twice as frequent as with the smaller inoculums. Thus, of the 68 strains inoculated heavily, 32 grew at 4 to 8° C. in blood and usually reached numbers which would probably be lethal upon transfusion. Nearly all strains grew in blood eventually at room temperature or in the incubator. Frequently, an initial reduction in plate counts was observed during the first few days of incubation at 27° and 37° C. Proteus grew fastest at all three temperatures of all warm-isolated species tested in both blood and broth. Coagulase negative staphylococci, *E. coli,* and *A. aerogenes* did not frequently grow and most of the strains of these species died in blood in the refrigerator. Nearly all of these gram negative bacilli produced visible growth in broth in the refrigerator. On the other hand, only 10 of 22 strains of a staphylococci grew visibly in broth in the refrigerator. It would appear, therefore, that blood suppressed growth or killed large inoculums of *E. coli, A. aerogenes,* and *P. aeruginosa* in the refrigerator since almost all of them could grow in broth at 4 to 8° C. Staphylococcus, on the other hand, frequently could not grow at 4 to 8° C. regardless of the media, so that the bactericidal or bacteriostatic action of blood was not a necessary factor for restricting development of that species in the refrigerator.

D. *The susceptibility of cold-growing bacteria to antibiotics in broth*

The results summarized in Table IV indicate that at 4 to 8° C. oxytetracycline, chlortetracycline, and polymyxin B were the most effective of the antibiotics. Chlortetracycline, an unstable antibiotic at 37° C., was more effective in the cold.

There was no difference between the effectiveness of polymyxin B at cold and warm temperatures. The higher of the two concentrations of chloramphenicol was less effective at cold temperatures than at warm temperatures.

E. *The susceptibility of cold-growing bacteria to antibiotics in blood*

The results are summarized for the two tetracycline antibiotics in Table V. Results indicate whether or not there was inhibition of growth in three weeks for refrigerated blood and at 36 hours for blood stored at 27° C.

Blood containing chlortetracycline almost never permitted growth at 4 to 8° C. Chlortetracycline occasionally permitted growth of cold-isolated bacteria (15 per cent) at 27° C. but not of warm-isolated bacteria. Oxytetracycline almost never permitted growth at either temperature and polymyxin B, tested only for its effectiveness in the cold, suppressed 92 per cent of bacteria. When no growth occurred in bloods containing antibiotics, the bacteria were usually reduced in number and sometimes sterilized in three weeks or less.

F. *The toxicity of cold-growing bacteria*

The toxicity of living bacteria in rabbits: The results are summarized in Table VI. The maximum numbers of *Achromobacter* or *Pseudomonas* which could be given in transfused blood without

TABLE VI

Toxicity (acute pathogenicity) of living gram negative bacilli for rabbits when transfused in 7.0 ml. of homologous rabbit blood *

Bacteria	Maximum sublethal dose	Maximum fever of any rabbit (*degrees Fahrenheit*)	Bacteria per ml. rabbit blood at 3 hours		Pathologic changes	
			Lethal dose	Sublethal dose	From lethal dose	From sublethal dose
A. aerogenes 3	10^9	4.5	I	3,960	0	NRT; MHA
A. aerogenes 1	10^9	2.8	862	3	0	PA; NRT
A. aerogenes 2	10^9	3.5	21,600	320–2,000	GT; NRT	HA; PA
E. coli 1	10^8	2.6			MHA	
E. coli 3	10^8	4.8		5–20	0	NRT; FRT
E. coli 2	10^7	3.4	2,500	47	NRT	
E. coli 4	10^8	3.5	510	13	NRT	NRT
P. aeruginosa A	10^{10}	4.0	I	164	GT	PA; HA
P. aeruginosa B	10^9	4.0	192	2–28	0	0
P. aeruginosa 1	10^9	3.9	306	6–220	0	NRT
P. aeruginosa C	10^7	4.0	49	6	0	0
Pr. vulgaris 11	10^8	4.0			FNS	0
Pr. vulgaris 19 A	10^8	4.1	2,000			
Pr. vulgaris R	10^9	4.3	3,665	88	NRT	0
Pr. vulgaris A	10^8	4.4	150	0	0	0
Coliform Hunt	10^{10}	4.4		200		
Coliform Bcx	10^8	4.4	104	12		
E. Freundii G	10^8		320	0		
Coliform G 2X	10^8	4.2		0–5		
Achromobacter 23	10^9	3.7	126	13–26		
Achromobacter 13A	10^{10}	2.5	3			HA
Achromobacter 9	10^8	4.0	810	7–134	MHA	FNS
Achromobacter SC	10^9	3.5		5		NRT
Achromobacter 250E	10^9	4.1	520	3–21	0	
Pseudomonad 29B	10^9	5.8	4	0	0	
Pseudomonad 20A	10^8	3.7	3	2	FNS	NRT
Pseudomonad 30A	10^{10}	4.4		330		
Pseudomonad 17B	10^{10}	4.5		152		
Pseudomonad 14B	10^8	5.0	168	40–60	FNS	

* Bacteria were added to blood immediately before transfusion. Living rabbits sacrificed at one week.
NRT = necrosis renal tubules; PA = pulmonary abscess; HA = hepatic abscess; M = miliary; FNS = focal necrosis of spleen; GT = glomerular thrombi; I = innumerable.

Dr. C. V. Weller assisted the authors in making these histopathologic interpretations.

TABLE VII

L.D./50 of crude bacterial endotoxins for mice (milligrams)

Isolated at 37° C.			Isolated at 4° C.			
P. aeruginosa	21	1.48	Pseudomonad	(dust) 14		1.11
	A	.417		(dust) 30A		1.45
	P	.417		(feces) 29B		.884
	B	.264		(dust) 20A		.845
E. coli	G	.368	Achromobacter	(feces) 106A		.312
	4	.361		(feces) 12C		1.87
	2	.350		208A		.675
	C	.557		(feces) 13A		1.85
				(feces) 23		.992
A. aerogenes	1	.061		(feces) 81		1.40
	N	.836				
	B	.739				
	3	.999				
Pr. vulgaris	B	1.53		Mean		1.14
	M	.850				
	S	.884				
	11	1.32				
Intermediate Coliform	H	1.52				
	2	1.65				
	B	.981				
	3	.661				
Mean		.843				

killing rabbits ranged from 10^8 to 10^{10} bacteria. The maximum sublethal numbers of coli-aerogenes, *P. aeruginosa,* and *Pr. vulgaris* were about the same. Lethal and sublethal numbers of the cold-isolated bacteria, pseudomonads, and achromobacters produced changes similar to those seen in animals receiving strains of coli-aerogenes, *P. aeruginosa,* and *Pr. vulgaris.* These consisted of necrosis of renal tubular epithelium, focal necrosis of the spleen, and generalized abscess formation. Many animals were free of pathologic changes. It is of interest that bacteremias at 3 hours in dying animals were more intense after transfusion of the warm-isolated bacteria than of the achromobacters and pseudomonads.

The chief findings in dying animals after transfusion with contaminated blood were severe weakness, fever, diarrhea, and a marked tendency to bleeding from the intestine and elsewhere. These findings were nearly identical in rabbits transfused with contaminated blood regardless of whether the contaminants were cold-growing or warm-growing bacteria.

The amount of growth in refrigerated blood required for lethal numbers of bacteria to develop from sublethal inoculums of cold-isolated and warm-isolated bacteria was to approximately 10^8 to 10^{10}, or the same as the lethal numbers after suspension of the bacteria in blood immediately prior to transfusion. Hence, it would appear that the toxic effects of transfused blood may depend on the number of gram negative bacilli; and that by-products of growth may not seriously influence the total toxic effect.

The toxicity of crude endotoxin in rabbits and mice: Various amounts of crude endotoxin from an achromobacter and a pseudomonad, both isolated at 4 to 8° C. produced essentially the same toxic effects in rabbits as equal doses of crude endotoxin prepared from a strain of *E. coli.* Doses of 10 to 15 mg. per Kg. of any of the three toxins killed all rabbits tested in 3 to 20 hours. Before death the rabbits displayed fever, diarrhea, weakness, and rectal bleeding. Doses of 5 mg. per Kg. or less led to diarrhea, listlessness, and fever but the rabbits survived.

In Table VII, the amount of crude endotoxin required to kill in 24 hours 50 per cent of inoculated mice is given for large numbers of cold-growing and warm-growing gram negative bacilli. The results indicate that the cold-isolated bacteria are nearly as toxic for mice and rabbits as the common enteric bacteria.

The total bacterial nitrogen concentration va-

ried from .10 to .14 mg. per mg. of crude endotoxin. The constancy of bacterial nitrogen from strain to strain indicates that equivalent amounts of bacterial products were being compared and that variations in toxic activity did not result from dilution of some endotoxins by non-bacterial products. Instead, the variations in toxicity would be expected to result from differences in potency of a given endotoxin, or from differences in total bacterial content of endotoxin.

G. *Protection against the toxic effects of gram negative bacilli*

Protection of recipient animals from contaminated transfused blood: The results in Table VIII disclose that the presence of antibiotics suppressed or destroyed the contaminants in transfused blood and completely prevented the bacteremia and severe *in vivo* effects observed in the controls. All control animals, receiving contaminated bloods without antibiotics, suffered fatal or nearly fatal reactions.

Protection by cortisone and antibiotics against crude endotoxin: In Table IX are the results of treating rabbits with cortisone after inoculation of lethal amounts of crude endotoxin from a pseudomonad, an achromobacter and a strain of *E. coli*. No protection by cortisone was observed among rabbits given *E. coli* toxin, but 14 of 15 cortisone treated rabbits survived the toxic effects of the two other toxins given in amounts which killed 13 of 15 controls. It is concluded that cortisone protected rabbits from the lethal effects of the crude toxins prepared from the two cold-growing bacteria.

TABLE VIII

Protection against bacterial transfusion reactions by antibiotics in contaminated blood (20 mcg./ml.) transfused into rabbits after 15 days refrigeration *

Bacteria	Total number of bacteria: Original inoculum	Total number of bacteria: Given in transfusion	Blood culture *Bacteria/ml.*	Antibiotic	Outcome
Coliform	10^4	10^{10}	—	None	Died 12 hrs.
2Z		10	0	A	Normal
		10	0	T	Normal
Coliform	10^4	10^{10}	I	None	Died
BCX		10^8	0	A	Normal
		10^8	0	T	Normal
Coliform	10^4	10^7	—	None	Died 5 hrs.
H		0	0	A	Normal
		0	0	T	Normal
Achromobacter	10^5	10^8	91	None	Died 12 hrs.
81		10^2	0	A	Normal
		10^4	0	T	Normal
Achromobacter	10^5	10^{10}	I	None	Died 10 hrs.
23		10^4	0	A	Normal
		10^4	0	T	Normal
Achromobacter	10^5	10^{10}	2,970	None	Died 12 hrs.
9		10^5	0	A	Normal
		10^5	0	T	Normal
Pseudomonad	10^8	10^{10}	100	None	Died 20 hrs.
29B		10^7	0	A	Listless
		10^2	0	T	Normal
Pseudomonad	10^5	10^8	12	None	Sick 3 days
14B		0	0	A	Normal
		10^8	0	T	Normal
Pseudomonad	10^5	10^{10}	106	None	Died 4th day
20A		10^5	0	A	Normal
		10^5	0	T	Normal

* A = chlortetracycline, T = oxytetracycline, I = innumerable.

TABLE IX

Results treating rabbits with intramuscular cortisone (15 mg./Kg.) at 24 hours and again at one hour in advance of intravenous injection of crude endotoxin (approximately 2 m.l.d.)

Crude toxin	Dose of toxin (mg./Kg.)	Number of rabbits surviving up to 24 hrs. Treated	Not treated
Pseudomonad 29B	20	5/5*	0/5
	15	5/5	2/5
E. coli G	12.5	0/5	0/5
	16	0/5†	0/5
Achromobacter 106A	25	4/5	0/5

* Received additional cortisone (15 mg./Kg.) 48 hours before toxin.
† Only received cortisone one hour before toxin.

In Table X, similar protection against crude endotoxin is seen in mice pretreated with cortisone but not in mice receiving cortisone at the same time the toxin was injected or afterwards. There is a suggestion in Table X that the tetracycline antibiotics also provided protection when given in advance of endotoxin. There was no sign of protection when the antibiotics were given at the same time, or after, the crude toxin.

DISCUSSION

Each of the two types of bacterial contaminants of banked blood presents a separate problem. First there are the cold-growing contaminants. These seem to be remarkably well suited for endangering the recipients of banked blood. Not only do they inhabit the premises and personnel of a blood bank, but they resist the bacteriostatic action of blood to such an extent that even small inoculums multiply heavily in the refrigerator, and sometimes at room temperature. These cold-growers are gram negative bacilli whose toxic potency is nearly equal to that of the endotoxins of the common intestinal gram negative bacilli.

The second type, which are classified as warm-growing contaminants, have less ability to grow in refrigerated blood, but grow better in blood at room temperature than the cold-growers. In Table XI, which lists the bacteria responsible for the violent transfusion reactions from contaminated blood reported to date, there are eleven, which are probably warm-growers, and seven, which would be expected to be cold-growers according to the present definition. One of the strains of *E. freundii* might have fallen into either group. These reports, therefore, suggest that the two types of contaminants are of nearly equal importance in leading to bacterial transfusion reactions.

Once blood becomes contaminated, the possibility of a serious reaction is not great because most contaminants are non-toxic gram positive bacteria which do not readily survive in refrigerated blood (7). Reactions will occur only when gram negative bacteria are stored in the cold or stand at room temperatures long enough

TABLE X

Protection of mice from lethal effect of crude endotoxin

Crude endotoxin	Dose (mg.)	Mortality rate in 24 hours: Controls	Cortisone 5 mg. 8 hrs. before toxin	Cortisone 5 mg. With toxin or later	Antibiotics* 2 mg. 8 hrs. before toxin
E. coli 4	.8	9/20	0/20		
	1.6	55/60	3/20	57/60	
A. aerogenes 3	5.0	20/20		20/20	
	2.5	36/40	7/20	34/40	11/20 (A)
Pr. vulgaris 11	5.0	20/20	8/20	17/20	6/20 (T)
P. aeruginosa	1.0	19/20	6/20		5/20 (A)
Achromobacter 13A	1.0	11/20	3/20		9/20 (A)
Totals		170/200	27/120	128/140	31/80

* A = chlortetracycline; T = oxytetracycline.

TABLE XI

Identification of bacteria reported to have been associated with severe bacterial transfusion reactions; of the 19 strains, 4 were said to be incapable of growth at 37° C. (Pseudomonas [5], [4])

Number of reactions associated with each bacterial species		Reported by
E. coli	3	Pittman (5); Officer (15)
"B. coli aerogenoides"	4	Whitby (2)
E. freundii	2	Braude *et al.* (3); Pittman (5)
Paracolon aerogenoides	2	Borden and Hall (1); Pittman (5)
Alcaligenes fecalis	1	Pittman (5)
Achromobacter sp.	1	Borden and Hall (1)
Pseudomonas sp.	5	Whitby (2); Pittman (5); Stevens *et al.* (4)
Intermediate Coliform	1	Braude *et al.* (3)
Total	19	

to grow to toxic proportions. It is likely that heavy growth at cold temperatures would result after contamination of banked blood with either a few cold-growers or numerous warm-growers, or at room temperature with small numbers of either type of gram negative contaminants. Precise limits on the period of storage or standing which would avoid heavy growth in contaminated blood cannot be given. It has been established in the present experiments, however, that the logarithmic growth phase is decelerated at cold temperatures so that a week or more is required for heavy bacterial growth in refrigerated blood. At room temperature, on the other hand, the typical steep logarithmic phase begins in about 6 hours in blood containing the usual inoculum of both cold and warm-growers, and by 24 hours, growth usually reaches lethal proportions (7). From these observations, it is clear that a minimum of standing in the refrigerator or donor room will reduce the hazard of bacterial reactions. Blood from banks with a turnover of most bottles within a few days should threaten fewer recipients with bacterial reactions than blood from banks which keep most bottles for more than one week. If a hospital cannot arrange for all bloods to be examined for bacterial contamination by direct smear at the time of transfusion, it should do so at least for those bloods refrigerated for longer than one week.

There is a possibility that gram negative bacilli may be so numerous at the time of contamination that their growth is unnecessary for the blood to produce a violent reaction. Reactions from such heavy contaminations have in fact been observed experimentally (7) and suspected clinically (3). They would be due to gross uncleanliness and negligence and could hardly be prevented by any known precaution once contamination had occurred. The only form of contamination from which the recipient can be confidently protected by precautionary measures is that in which growth is still required for the blood to assume dangerous properties. The routine use of either chlortetracycline, oxytetracycline, or polymyxin B in concentrations of 10 mg. per pint can prevent large sublethal inoculums of either cold-growers or warm-growers from multiplying in human blood at both refrigerator and room temperature (Table V). Transfusion of contaminated blood containing that concentration of chlortetracycline or oxytetracycline during refrigeration produced no harm to animals under conditions which resulted in the death of nearly all control animals (Table VIII). Such minute quantities of either tetracycline antibiotic would probably be harmless to the recipients. Patients who receive blood transfusions are frequently given a variety of antibiotics for prophylactic and therapeutic reasons. The small amount in the transfused blood would be negligible compared to what he is likely to be receiving otherwise. The routine use of the tetracycline antibiotics in all banked blood has been suggested by Stevens and his co-workers (4) and should be seriously considered especially under those difficult conditions of storage or handling which may be encountered in civil catastrophes or war. Blood alone is bacteriostatic against only some of the contaminants (Table V); the same is true of antibiotics (Table IV). But together they are remarkably effective in preventing growth of nearly all warm-growing or cold-growing types of contaminants. It is of interest that the tetracycline antibiotics have also proved the most effective inhibitors of growth of the natural mixed bacterial flora of fish and meat stored at temperatures between 0° C. and 21° C. (12).

If growth of gram negative bacilli is not prevented before transfusion, they may kill the recipient. The mechanism of killing is related to the action of the bacterial endotoxin and not necessarily to invasive properties of the bacteria. Thus, rabbits transfused with lethal numbers of cold-growing bacilli (pseudomonads or achromobacters) had sometimes cleared their blood al-

most entirely of bacteria at the time of death (Table VI). These animals had apparently died of the effects of endotoxin rather than those of an overwhelming septicemia resulting from *in vivo* growth of the bacteria. Similar clinical phenomena after transfusions of heavily contaminated blood have been seen in patients who were in profound shock or died despite the absence of demonstrable bacteria in the blood or tissues (1, 3, 4). The chief source of toxin from gram negative bacilli multiplying in refrigerated blood appears to be the endotoxin within the bacilli; other toxic products are apparently not manufactured in significant amounts during growth in blood. This conclusion is based on the observation in this study that lethal numbers of viable bacteria, resulting from multiplication in blood, are approximately the same as lethal numbers after suspension of bacteria in blood immediately prior to transfusion. Geller and Jawetz (13) have recently presented evidence that in mice part of the bacterial multiplication involved in reaching lethal doses of endotoxin may take place *in vivo*.

Once transfusion of heavily contaminated blood has occurred, it is difficult to provide protection and the recipient usually dies. In experimental animals challenged with endotoxin, the most consistently effective protective measure has been prior treatment with cortisone. In these studies, protection from cortisone was observed not only in rabbits given crude endotoxin obtained from cultures of *Achromobacter* and pseudomonads, but also in mice challenged with crude endotoxins of *E. coli, A. aerogenes, Pr. vulgaris, P. aeruginosa,* and *Achromobacter*. Similar observations have been reported earlier by Boyer and Chedid (14). Antibiotic treatment of animals given crude endotoxin was less consistently effective in these experiments than treatment with cortisone. In the only report of successful treatment of an overwhelming bacterial transfusion reaction, the patient received not only antibiotics and cortisone, but nor-epinephrine as well (3). In fact, the use of nor-epinephrine in large quantities for several days appeared to be the most valuable measure of all those employed in the successful treatment. This drug was essential for overcoming the state of severe and persistent hypotension which is the most striking clinical effect of transfusing contaminated blood.

Perhaps the crucial point in establishing successful treatment is prompt recognition of the syndrome. Unfortunately the patient who has received contaminated blood may not appear gravely ill during the first 12 hours after the transfusion and there may be few outspoken signs to call attention to the serious state of hypotension. The syndrome is most quickly identified by examining a direct smear of the blood from the transfusion bottle. Gram negative bacilli will be found in every field, and treatment must be instituted at once without waiting for identification of the bacteria.

When attempts are made to recover the contaminating bacteria, cultures should be incubated at 27° C. as well as 37° C. because an occasional strain of cold-growing bacteria cannot grow at the usual incubator temperatures. All but 4 of the 19 reported strains listed in Table XI could be cultivated at 37° C. These organisms, which cannot grow at 37° C., represent only a small minority of cold-growing bacteria and are less capable of vigorous multiplication in refrigerated blood than those cold-growing bacteria which can multiply well at 37° C. It is not surprising, therefore, that they have been incriminated only infrequently as the cause of transfusion reactions.

The cold-growing bacterial contaminant of blood must be regarded as a newly recognized type of pathogenic microorganism. It is pathogenic because of its capacity to produce both clinical and experimental disease; but lacking any significant ability to multiply in the host, it exerts its pathogenicity entirely through an endotoxin which is produced outside of the body and injected intravenously. The vector is thus man himself who confers upon the bacteria the attribute of communicability. The cold-growing bacteria may be compared in this sense with *Clostridium botulinum* which also lacks invasive capacities and depends entirely for its pathogenicity on toxic properties. The great point of difference between the two microorganisms, however, is that the toxicity of *Cl. botulinum* is due only to its potent exotoxin. It possesses no endotoxin.

SUMMARY

Bacteria capable of heavy growth at refrigerator temperatures (4 to 8° C.) can be easily re-

covered in the blood bank from potential sources of contamination for stored blood. The majority of these cold-growing contaminants are gram negative bacilli which have been identified as pseudomonads, coliforms, and achromobacters. Their toxic potency is nearly equal to that of the endotoxins of common intestinal bacilli. Because even small inoculums can resist the bacteriostatic action of blood and multiply quickly to lethal numbers in the refrigerator, these cold-growing bacilli constitute a grave threat to recipients of contaminated banked blood. They may be consistently suppressed, however, in blood containing such minute amounts of tetracycline antibiotics that risk to the recipient from the antibiotics is unlikely.

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EXPERIENCES WITH RENAL HOMOTRANSPLANTATION IN THE HUMAN: REPORT OF NINE CASES[1]

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We report in this paper studies on nine patients in whom homologous kidney transplantation was performed. A preliminary report of some of these cases has already been made (51, 58).

All of the recipients were patients in the terminal stages of severe, advanced renal failure. The risks were fully discussed with the patients and their families, and the uncertain outcome made clear.

In several instances the function of the transplant was maintained for a surprisingly long period of time. All of the patients ultimately succumbed to their disease.

It was our aim in this investigation to study the subject of homotransplantation of the human kidney—not to offer a therapeutic procedure. We agree with Dempster (33) that "it is quite out of the question that kidneys should be homotransplanted in man just in case a permanent survival might be obtained." Nevertheless, we felt that this study should be undertaken for the following reasons:

1) It is important to know if the underlying chronic renal disease of the patient can produce changes which are able to influence—either adversely or favorably—the course of the homotransplant. It is difficult to simulate human renal disease accurately in the experimental animal.

2) It has been generally accepted that the higher an animal is in the phylogenetic scale, the more highly developed is its ability to form antibodies, and thus, presumably, to destroy homografted tissue. This thesis has recently been supported by Dempster (33). No conclusive study of the fate of human renal homotransplants was available, however. It seemed desirable to define the course of these transplants, and to see how the rejection of the transplanted kidney in man compared to that in lower animals.

3) It was felt that observations of the biologic response to kidney homotransplantation in man might yield information that could not be obtained by animal experimentation alone. Data might be acquired which would suggest new lines of approach to the homotransplant problem and these could then be further evaluated at the level of the laboratory animal.

Our results appear to indicate 1) that the disease of the patient can, under some circumstances, influence the course of the transplant, 2) that it is possible to perform renal homotransplants in the human that survive and function for far longer periods of time than those reported for any experimental animal, and 3) that the rejection of the human homotransplant is not always comparable to that seen in the dog.

The development of the artificial kidney (63), more than any other single thing, led to the undertaking of our study of human homotransplantation at this time. A modified Kolff kidney was constructed and put in operation at the Peter Bent Brigham Hospital in 1949 (99). By this means it became possible to prepare seriously ill uremic patients for surgery, and to maintain them in the post-operative period, so that it was feasible to perform transplantation in the terminal stages of chronic renal failure.

It would still not be tenable to attempt renal homografting in the human unless every effort were made to render the procedure as safe as possible, and to subtract nothing from whatever renal function remained to the patient. It was also imperative that the transplantation be performed in such a way that the function and course of the grafted kidney could be charted as accurately as possible.

We chose to transplant the kidneys into the

[1] Aided by grants from the Bristol-Myers Pharmaceutical Company; The Lasdon Foundation; The National Institutes of Health; and Eli Lilly & Company.

thigh of the recipient for the following reasons: 1) It is preferable in a uremic patient to add a third kidney, rather than to remove one of the recipient's own kidneys and substitute a transplant; 2) It is less traumatic to place the kidney in the thigh than in the renal fossa; 3) A skin ureterostomy permits collection of urine directly from the transplant and facilitates comparison of its function with that of the other two kidneys; 4) The vascularity of the ureter is a good index to the circulation of the transplant, because the blood supply of the transplanted ureter comes entirely from the renal vessels; 5) If the ureter sloughs, no serious complication ensues, as it might in intra- or retro-peritoneal locations; 6) If the transplanted kidney dies, or becomes seriously infected, the complication can be detected sooner than would be the case with a transplant in the renal fossa, and the kidney can be removed more readily; 7) It is much easier to biopsy the kidney when it is placed in the thigh.

The location of the transplant in the thigh has the disadvantage that the circumference of the thigh has to be increased by skin grafting to receive the kidney without tension on the overlying tissues. This necessitates opening up planes of dissection which are often very vascular and which, in the uremic patient, sometimes continue to bleed despite strenuous measures to provide hemostasis.

REVIEW OF THE LITERATURE

There have been a great many papers written on the general problem of tissue homografting. Recent reviews and bibliographies are available which cover various aspects of the transplantation problem (11, 39, 43, 83, 84, 92, 108, 121, 123, 126, 127, 137). No attempt will be made here to do more than briefly summarize the more pertinent studies of renal homotransplantation. Reference will be made in the discussion to certain papers which deal with questions of immunity and transplant pathology, and that are considered relevant to the analysis of the cases reported in this communication.

Renal homotransplantation in experimental animals

It is difficult to evaluate much of the earlier work because details of urinary secretion and composition, experimental protocol, and studies of pathology are often wanting. The period of homograft survival is sometimes considered to be the time of stoppage of urinary secretion (accurate collections have seldom been achieved), and sometimes the death of the animal, if bilateral nephrectomy was carried out at, or subsequent to, the transplantation.

Ullmann, in 1902 (128) was the first to carry out renal auto-, homo-, and hetero-transplantation, using prosthetic tubes to make the anastomoses. He made transplants from one dog to another, and from a dog to a goat, placing the kidney in the neck. No details of urinary secretion were published.

Carrel, in 1902 (18) developed a new technique for vascular anastomosis, and performed several auto transplants to the neck, noting that they secreted clear urine. All transplants were ultimately destroyed by infection. Carrel and Guthrie in 1905 (19) compared the function of a renal homograft done three days before to the normal kidney. They found the rate of secretion to be greater than normal, but the excretion of metabolites to be less than normal. In 1906, the same authors developed the "patching method" for anastomosis of small vessels (20). They made brief mention of two transplants made in cats by this technique. In 1906 they also reported an experiment in which they had transplanted two kidneys from one dog to another by an "in mass" technique. The kidneys and surrounding tissues were removed en masse with segments of aorta and vena cava which were then anastomosed in continuity to the aorta and vena cava of the host (21). Both kidneys of the host were removed. The urine from the transplants was said to be within normal limits on the 8th day. The end result of this case, a 10-day survival, was published two years later (22).

In 1908, Carrel (22) reported the results of transplantation in mass of both kidneys (the host's kidneys being removed) to 14 cats. Five died in the immediate post-operative period. Seven of the remaining cats died within 14 days. Two animals lived for 31 and 36 days, respectively. The first of these two animals was noted to have an enlargement of the transplants and marked albuminuria on the 18th day. At autopsy an acute interstitial nephritis with plasma cell infiltration was found, and was noted to be more marked in the cortex than in the medulla. In the second cat swelling of the transplant and albuminuria was noted on the 16th day. An exploratory laparotomy on the 18th day revealed the transplanted kidneys to be edematous and large. At autopsy on the 36th day a marked calcification of the aorta and other vessels was noted (23). The kidneys were said to have secreted urine up to the day of death.

Floresco in 1905 (46) performed renal homotransplants to the inguinal area, the neck, and the renal fossa in dogs. He concluded that the renal fossa was the most suitable location, the inguinal area the least. The average survival of the transplants was about 3 days.

Villard and Tavernier in 1910 (130) and Villard and Perrin in 1913 (131) homografted the kidney in nine instances. The longest time any of the transplants secreted urine was 8 days.

Avramovici in 1924 (4) reported doing renal heterotransplants from cat to dog en masse, taking out both

the recipient's kidneys. Two of these cases were said to have survived 49 and 58 days, with normal renal secretion. At autopsy the transplanted kidneys were said to appear normal. No histological studies or urine analyses were done. Homotransplantations, en masse, were done in 11 dogs, all recipients having bilateral nephrectomies. One dog was said to have lived 61 days, dying of bronchopneumonia. Another lived 73 days. No histological or urinary studies were reported. In six additional experiments, bilateral renal homografts were done in two stages. Three of these animals were said to have survived 33, 44, and 58 days. When transplanting only one kidney (leaving one of the host's kidneys *in situ*) survivals of 41, 56, and 60 days were obtained. The only mention made of microscopic studies was in the animal which survived 56 days. The transplanted kidney was said to show glomerular lesions, alterations of the tubular epithelium, and an inflammation—not too marked—of the interstitial tissue.

Avramovici also performed transplantations of kidneys which had been preserved in the cold for varying periods of time up to 30 hours. One transplant which had been thus preserved for 8 hours was then homotransplanted, one of the host's kidneys being removed. Fourteen days later the other kidney of the host was removed. The animal was said to have lived 36 days more.

Renal homotransplantations in experimental animals as successful as those reported by Avramovici have not been achieved by any other workers before or since. The absence of microscopic and urinary studies, added to the lack of confirmation by others, makes it difficult to attribute much importance to these findings.

Dederer, 1920 (28, 29), performed a homotransplant of a kidney from one puppy to another of the same litter, making the anastomosis in the neck and leaving both of the recipient's kidneys intact. The animal died of distemper on the 26th day. The only function test done was to inject PSP dye, which was said to have been excreted by the transplanted kidney, following injection, on the 26th day.

Williamson, 1923 (135) and 1926 (136), using dogs and goats, confirmed Carrel's findings that whereas autogenous kidney transplants would maintain the life of the animal for months after removal of the other kidney, homologous transplants functioned only for a period of days. The histologic picture said by him to follow homotransplantation in the dog was that of acute atypical glomerulonephritis with lymphocytic infiltration, followed by general acute nephritis and ascending infection. If the transplanted kidney was allowed to remain in place after anuria developed, there was a tendency for it to be replaced by fibrous tissue. He concluded that the failure of homologous kidney transplants seemed attributable to a biologic incompatibility between the donor and recipient. He felt that the agent causing this was blood-borne, attacking first the glomeruli, and then the tubules.

Ibuka, 1926 (61) reviewed the literature on renal homotransplantation up to that date. He did a series of autotransplants to the neck in dogs, to demonstrate that these were capable of functioning for months. He then did 14 homotransplants by the same technique. Two were technical failures, and 4 succumbed to immediate post-operative sequellae. The remaining 8 cases were carefully followed, and analyses were performed on the urine. The transplants secreted urine for from 1 to 5 days, averaging 3 days. They stopped secreting before infection set in, and in spite of having an adequate blood flow and maintaining a patent ureter. Microscopically they showed an acute nephritis with lymphocytic and plasma cell infiltration and tubular destruction. The glomeruli appeared fairly normal.

Holloway, 1926, (54) found that homotransplants remained viable only two to four days, the destructive process starting in 24 hours.

Wu and Mann, 1934, (139) made a microscopic study of the day to day progress of auto- and homo-transplants in the dog. The transplants functioned alike until 1) they were removed, 2) the animal died, 3) thrombosis of one or both vessels occurred, or 4) pyelonephritis supervened. Some transplants never functioned. This was not due to thrombosis. Microscopically, monocytic interstitial infiltration and tubular necrosis were observed. The glomeruli still looked quite normal.

Parkinson and Woodworth, 1947, (105) performed renal transplantations in goats, and refer to them as heterotransplants, on the basis that the goats used were of different types. The kidneys were placed in the neck. Anastomoses were made with vitallium tubes. Autografts always developed such good collateral supply that they continued to function even when the vessels thrombosed. Homografts had an active arterial supply and venous return at one week, but had become soft, nearly gelatinous, and poorly demarcated. They secreted urine for 10 days. At 14 days they were dissolved.

Oudot, 1948 (104) cooled the kidneys to 4 degrees C. for periods up to 8 days before transplanting them. Infarction of the kidneys occurred immediately after transplantation. Payr tubes of "superpolyamides" were used as prostheses. Non-cooled renal homografts with cutaneous ureterostomies stopped functioning in 10 days. This was felt to be on the basis of a suppurative necrosis due to ascending infection.

Lefebvre, 1949, (74) showed that a homograft can clear urea. He reports one dog which lived for 21 days after homotransplantation, with a normal blood urea nitrogen for the first 19 days. The transplants were made in the neck, with vitallium prostheses. He also studied the effect of perfusing the kidney and storing it at low temperatures before transplantation (75). The transplanted kidney was observed only for short periods of time. He found that kidneys stored up to 24 hours were capable of showing a return of function after transplantation, as measured by diuresis, chloride output, return of oxygen consumption, excretion of bilirubin and urochrome, and urea output. All of these indices, except chloride output, showed the function to be below that

of a normal kidney. (Maximum urea concentration noted was five times that of blood).

Recently, Simonsen and his co-workers (119–121) and Dempster (30–34) have studied renal homotransplantation in dogs. Their work will be considered in some detail later on in the paper.

To summarize, most investigators have found that renal homotransplants in the experimental animal function for from 1 to 18 days, with an average of about 4 days. They cease urine secretion at a time when blood flow through the renal vessels can still be demonstrated. Histologically, the kidney which has stopped secreting shows interstitial edema and round cell infiltration and tubular destruction. The glomeruli remain relatively normal.

Renal homotransplantation in the human

On occasion an attempt has been made to use a human kidney homograft as a temporary aid to tide the patient over an episode of acute anuria. Voronoy, 1936 (132) transplanted a kidney into the groin of a patient with bichloride of mercury poisoning, but the patient died in 48 hours and no conclusions can be drawn from this experiment. Details are lacking. Landsteiner and Hufnagel in 1945 (70) transplanted a kidney from a cadaver to the brachial artery and cephalic vein of a young woman in acute renal failure. The patient's own kidneys began to resume function a few hours after the transplantation, and the transplant was removed after 48 hours, without having secreted any significant amount of urine.

Lawler, West, McNulty, Clancy, and Murphy in 1950, (72) removed a kidney from a woman with polycystic kidney disease and anastomosed the kidney from a cadaver to the patient's renal artery and vein. The ureter was anastomosed to that of the recipient. These authors injected indigo carmine dye parenterally on the 52nd day, and stated that it was observed to emerge from both ureters, but in poorer concentration from the side of the transplanted kidney. No other kidney function tests were done. At the time of removal of the transplant, 9½ months after implantation, the kidney was found to be 4 by 3 by 2 cm. in size, with absence of the ureteral and pelvic structures. It was not producing urine (73).

Servelle, Soulié, Rougeulle, Delahaye, and Touche (115, 116) reported one human homotransplant, and Dubost and his associates (35) reported two at a meeting of the Medical Society of Paris Hospitals in January, 1951. All transplants were made in the pelvis, and the vessels were anastomosed to the iliac vessels. The kidney transplanted by Servelle and his co-workers reached an output of 600 ml. by the 19th day, at which time the patient suddenly died. The two transplants reported by Dubost and his associates had been performed only 14 and 3 days before the meeting. The first kidney secreted a maximum of 230 ml. of urine in 24 hours, and the patient died on the 16th day (36). The second transplant secreted only a few milliliters.

Kuss, Tienturier, and Milliez are reported by Lawler, West, McNulty, Clancy, and Murphy (73) to have performed 16 homotransplants in the human, several of which have "functioned for several months," but in their publication (68) they describe only five, and in a personal communication to one of us (DMH) (69) Kuss reports having done only one more. Of the five published cases, one patient died on the operating table, and in one the graft was removed 48 hours after the transplantation. A third case developed necrosis of the ureter, and the transplant excreted small amounts of urine for only 18 days. The patient died on the 20th day. A fourth transplant developed necrosis of the ureter on the 20th day. The maximum output was 72 ml. in 24 hours, on the 10th day. The kidney was still excreting a few drops of urine when it was removed after 3½ months. In the final subject the maximal 24-hour output was 45 ml. The ureter became necrotic on the 20th day, and the patient died on the 35th day. An extensive infarction of the kidney was found at necropsy. The sixth case was said to have shown no better function (69). In these cases the transplant was placed in the pelvis of the recipient, and an anastomosis was carried out between the hypogastric artery and the renal artery, and between the external iliac vein and the renal vein. The ureter was brought out to the skin.

Recently, Michon and his associates (101) have reported a case in which the transplant functioned well for 23 days. More will be said about this case later.

In general, renal homotransplantation in the human has not been any more successful than in experimental animals.

METHODS

In eight of the nine cases reported herein the homologous kidney was transplanted into a pocket created in the medial aspect of the upper thigh. In all eight cases anastomoses were carried out between the deep femoral artery and the renal artery, and between the renal vein and the femoral vein. In one instance an end-to-end anastomosis was made between the superficial femoral vein and the renal vein, and in one case a similar anastomosis was performed using the common femoral vein. In six cases an end-to-side anastomosis was made between the renal vein and the common femoral vein, and this was found to be the most satisfactory method. In all cases the deep femoral artery was isolated for a distance of 7 to 8 cm., and all branches were ligated and divided. It was then ligated as far distally as possible, a bulldog clamp was placed on it proximally, just distal to the bifurcation of the common femoral, and it was divided just proximal to the tie. A site was selected along the isolated vessel which most closely conformed to the size of the renal artery, and it was again divided at this point, and end-to-end anastomosis with the renal artery was carried out. All anastomoses were done with 6-0 arterial silk, using continuous everting mattress sutures, and tying the suture to each of three or four interrupted stay sutures. The ureter was brought out

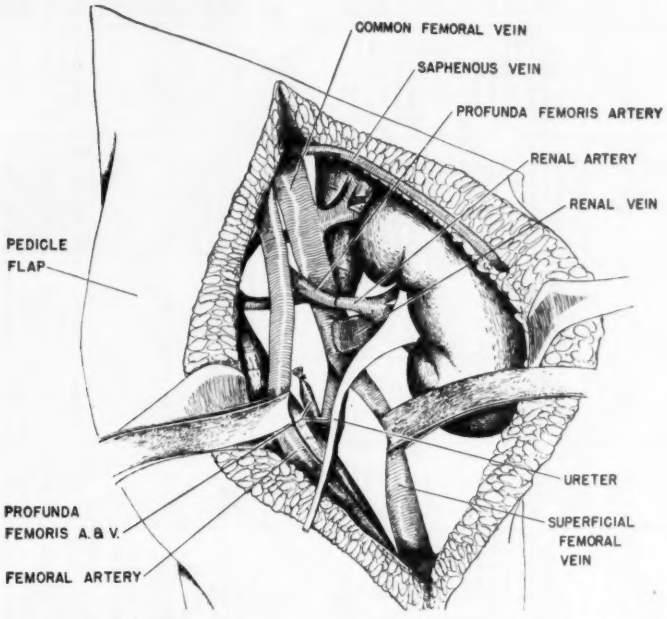


FIG. 1. THE METHOD OF PLACING THE RENAL TRANSPLANT IN THE RIGHT THIGH

The kidney is buried in a pocket created between the subcutaneous tissue and the muscles in the medial aspect of the thigh. The renal artery is anastomosed end-to-end to the profunda femoris artery, and the renal vein is anastomosed end-to-side into the common femoral vein. A double-ended pedicle flap is pulled over to help cover the kidney without tension on the skin. A split skin graft (not shown) is then applied over the lateral aspect of the thigh in the defect left by the moving over of the pedicle flap. The ureter will be brought out through a stab wound in the lower portion of the pedicle flap.

through a small incision in the skin. The relation of the vessels to the kidney is shown in Figure 1.

In the first two cases the kidney was placed in a space fashioned between the subcutaneous tissue and the fascia overlying the adductor muscles. This created a great deal of tension on the skin, and a slough of the skin developed. In five cases a double-ended pedicle of skin and subcutaneous tissue 30 by 15 cm. in size was raised off the fascia laterally, swung over medially to create a covering for the kidney without tension on the skin. A split thickness graft taken from the other leg was then used to cover the defect left laterally over the fascia lata. This worked very satisfactorily. In one case pieces of the rectus femoris and sartorius muscle were removed and end-to-end anastomosis with the deep femoral artery and common femoral vein was carried out over polyethylene tubes which were temporarily tied in place to re-establish circulation as rapidly as possible. This technique of vascular suture has been described by Hufnagel (56). The kidney was then placed in the defect left by the removal of the muscles, and the skin was closed over it without tension. No skin flap was used. A great deal of tension developed by the 5th day, however, because of hemorrhage from the cut muscles and edema of the leg, and the wound had to be reopened, and a relaxing incision made.

In eight cases heparin was used for varying lengths of time, and in six cases ACTH or cortisone or both were employed. Testosterone was given in every case. Various antibiotics were administered.

The donor kidneys were obtained from cadavers in six cases.[2]. Great care was taken not to injure the blood supply of the ureter. The kidneys were removed under strict aseptic conditions. In two cases the kidneys came from living donors and were essentially normal kidneys. These two cases were patients aged 2 and 33, the first with communicating hydrocephalus and the second with hydrocephalus caused by a block of the aque-

[2] In three of these the kidney was removed by Dr. Frank Collins, in one by Dr. Phillip Walker, and in two by Dr. Robert Desautels.

TABLE I

The nine cases reported here are summarized in this table

Case	Donor				Recipient								Result		
	Origin kidney / How treated	Kidney function in donor	Age, sex, disease of donor	Blood type	Blood type	Disease of recipient	Period anoxia ×plant	Operative technic	ACTH and cortisone	Heparin	Testosterone	Antibiotics	Complications	Hemodialysis	Function of transplant
No. 1 F. A. male 37 No. 8B438 Op. 3-31-51	Operative removal Not irrigated	Normal	Male—49 Carcinoma of lower end of ureter.	A+	A+	Chronic pyelonephritis, hypertension (190/100), uremia.	min. 70	Left renal fossa, spleen and left kidney removed. Splenic artery anastomosed to renal artery and renal vein to renal vein. Heat's ureter to pelvis of transplant.	*ACTH:* *day daily dose* 1-3 20 mgm. I-M 4 65 mgm. I-M 5-8 25 mgm. I-V 8-9 100 mgm. I-M 10 25 mgm. I-V 11 10 mgm. I-V 12 0 13 5 mgm. I-V	Heparinized 48° 200 mgm./D 5-9 D	100 mgm.—15th day 50 mgm./D 16-37 D	Penicillin® Terramycin® Streptomycin Chloromycetin®	Infection of flank wound. Secondary closure done 23rd day.	5 days pre-op. 1 day pre-op. 9 days post-op. 18 days post-op. 26 days post-op. 32 days post-op.	Difficult to assess function because of T-tube. Did not excrete dye or maintain patient. Died 37th day.
No. 2 M.P. female 30 No. 6B376 Op. 4-23-51	Cadaver Irrigated with saline	BUN elevated 25-45. Had received mercurial diuretics. 1-3+ protein PSP 50%	Male—48 Hypertension. Died during operation for hypertension.	O+	A+	Pyelonephritis, hypertension, chronic uremia.	150	End-to-end anastomosis profunda femoris artery with renal artery. End-to-end renal vein with superficial femoral vein. No pedicle graft. Right leg.	*ACTH:* 60 mgm./D I-V—48 hrs.; then *Cortisone:* 25 mgm./D for 7 D	160 mgm./D for 2 D	50 mgm./D for 7 D	Penicillin	Tremendous tension developed in wound due hematoma and edema. Explored 3rd day, removed 17th day. 6 days after removed hemorrhage occurred from eroded femoral artery. Plastic tube put in.	33 days pre-op. 2 days post-op.	Infarcted between 3-13th day. Removed 17th day. No function. Patient died 25th day.
No. 3 F. Z. female 43 No. 5A157 Op. 4-24-51	Cadaver Not irrigated	1+—4+ albumen in urine, apparently on basis congestive failure. BUN normal. Microscopic exam other kidney showed multiple small infarcts.	Female—47 Mitral stenosis. Died during operation for mitral stenosis.	B+	B+	Chronic pyelonephritis, hypertension, uremia, arteriosclerosis.	135	End-to-end anastomosis profunda femoris artery with renal artery. End-to-side renal vein into common femoral vein. No pedicle graft. Right leg.	*ACTH:* 100 mgm./D I-M—24 hrs. 80 mgm./D—18th-31st day *Cortisone:* 225-300 mgm./D for 18 D; then 100 mgm./D thru 28th day.	160 mgm./D for 14 D	200 mgm.—24° 50 mgm./D for 34 D	Penicillin	Skin sloughed over kidney.	47 days pre-op. 22 days pre-op. 57 days post-op.	Anuric 8½ days. Maximum function 18-22nd day. Output good 33 days. Output fair 53 days. Still excreting some on 70th day—biopsy at this time showed viable kidney. Patient died 99th day.
No. 4 B. B. female 52 No. 7B634 Op. 5-26-51	Cadaver Not irrigated	BUN normal 1+ protein	Male—21 Tetralogy of Fallot. Died 4th p.o. day following operation to widen pulmonary valve.	O+	O+	Polycystic kidney disease, pyelonephritis, hypertension, uremia.	200	End-to-end anastomosis profunda femoris artery with renal artery. End-to-side renal vein into common femoral vein. Pedicle graft swung. Right leg.	*ACTH:* 100 mgm./D I-M 8-20th D, then tapered 21-24th D. *Cortisone:* 300 mgm./D—7 D, then 150 mgm./D 8-13th D. Tapered off 14-18th D. 150 mgm./D—29-40th D. Tapered off. 41-44th D.	80 mgm./D for 6 D 100 mgm./D —6-14 D 80 mgm./D —15-24 D	200 mgm.—24° 50 mgm./D —25 D 50 mgm./D —66-90 D	Penicillin Terramycin® Chloromycetin® Aureomycin Sulfasucidine®	29th day got chill, fever to 104, decreased transplant output. By 37th day fever and output had improved.	2 times pre-op. 98 days post-op. 100 days post-op.	Anuric 11 days. Maximum function 13-28th day. Grad. declining function 29-65th day. Output dropped markedly 66-101st day. Removed 101st day.

TABLE I—*Continued*

Case	Donor				Recipient								Result		
	Origin kidney / How treated	Kidney function in donor	Age, sex, disease of donor	Blood type	Blood type	Disease of recipient	Period anoxia X-plant	Operative technic	ACTH and cortisone	Heparin	Testosterone	Antibiotics	Complications	Hemodialysis	Function of transplant
No. 5 D. P. female 18 No. 9A816 Op. 6-6-51	Operative removal / Not irrigated	Normal	Female—2½ Hydrocephalus. Kidney removed for subarachnoid-ureteral anastomosis.	O+	O+	Chronic glomerulonephritis, hypertension, (204/130) uremia, arteriosclerosis.	*min.* 55	End-to-end anastomosis profunda femoris artery with renal artery. End-to-side renal vein into common femoral vein. Pedicle graft swung. Ureter wouldn't reach skin, so polyethylene tube tied in ureter. Left leg.	*ACTH:* 100 mgm./D 1-M 8–15th D. Tapered 16–19th days. *Cortisone:* 300 mgm./D—8 days. Tapered 9–13th days.	100 mgm./D 2nd day 80 mgm./D 3–11 D	200 mgm.—24° 50 mgm./D —27 D	Penicillin Terramycin® Streptomycin Chloromycetin®	Infected hematoma developed rt. thigh (at site previous op.). Aspirated 27th day and often thereafter. On 29th day kinking of ureter with block of pelvis and infection developed in transplant. Explored and drained on 37th and 41st days.	None	1700 ml. urine excreted first 24°. 153 ml. second 24°, then relative anuria for 12 days. On 14th day kidney opened up. Max. function 14–26th D. Function declined 26–37th D, and almost no urine excreted after this. Patient died 47th day.
No. 6 B. L. male 4 No. 9B64 Op. 7-23-51	Operative removal / Not irrigated	Normal	Female—3 Hydrocephalus due tumor. Kidney removed for ventricular ureteral anastomosis.	O+	O+	Chronic pyelonephritis, uremia.	14	End-to-end anastomosis profunda femoris artery with renal artery. End-to-end renal vein and common femoral vein. Anastomoses done over temporary polyethylene tubes. Rectus and sartorius muscles partially removed. No graft. Left leg.	None	100–190 mgm./D—12 D 60–100 mgm./D—14–18 D	50 mgm./D 11–19 D	Penicillin Terramycin® Chloromycetin® Aureomycin	Hematoma and pressure on kidney. Exploration and relaxing incision on 19th day. Kidney removed 52nd day. Five days later patient exsanguinated from eroded femoral artery.	4 days post-op. 14 days post-op. 49 days post-op.	700 ml. urine excreted first 12 hours, then kidney became anuric and never opened up. Biopsied 19th day. Removed 52nd day. Patient died 57th day.
No. 7 D. G. female 28 No. 6E373 Op. 5-7-52	Cadaver / Irrigated ringers with albumen and penicillin	1+ to 2+ protein. BUN normal. Aplastic kidney left, slightly hypertrophic kidney right. Aberrant vessel to right lower pole.	Female—33 Intra-auricular septal defect, diastolic hypertension. Died during operation to close defect.	B+	O+	Chronic pyelonephritis, hypertension (200/105), congestive heart failure, uremia.	200	End-to-end anastomosis profunda femoris artery with renal artery. End-to-side renal vein into common femoral vein. Pedicle graft swung. Right leg.	None	160 mgm./D for 2 D	50 mgm./D 12–19 D	Penicillin	Bleeding beneath skin flap and graft—no pressure, however. Severe hyperkalemia.	2 times pre-op. 4th day p.o.—peritoneal lavage 5th day p.o.—dialysis artificial kidney 13th day p.o.—peritoneal lavage 17th day p.o.—perit'l lavage.	No significant function. Kidney removed 17th day—totally infarcted. Patient died 19th day.
No. 8 O. F. female 57 No. 7E213 Op. 9-22-52	Cadaver / Not irrigated	Donor had congestive failure. Urine 1–2+ protein, some hyaline casts. BUN 18.	Male—33 Ruptured sinus of valsalva, and intraventricular septal defect. Died during cardiac operation.	O+	O+	Periarteritis nodosa, with destruction of kidney and anuria.	275	End-to-end anastomosis profunda femoris artery with renal artery. End-to-side renal vein into common femoral vein. Pedicle graft swung. Left leg.	100 mgm./D I-M for 5 D; then 25 mgm. for 8 hrs. I-V q. D 6–7th D; then 40 mgm. (gel) q. D 7–19th D. Tapered off 20–22nd D.	90 mgm.—18 hrs.; 160 mgm./D 1–7th D; tapered off 7–10th D.	200 mgm.—24°; 50 mgm./D 12 D; then 50 mgm. q. 2 D 13–38th D.	Penicillin Streptomycin Terramycin®	Hemorrhage under split graft-lifted this off. Slight wound infection.	28 days pre-op. 19 days pre-op. 7 days pre-op. 6 days post-op. 16 days post-op.	No significant function. Patient died 38th day. Transplant showed marked glomerulonephritis.
No. 9 G. W. male 26 No. 7E503 Op. 2-11-53	Cadaver / Not irrigated	Donor had congestive failure. No protein. BUN normal.	Female—37 Mitral and aortic stenosis. Died during cardiac operation.	A+	A+	Chronic glomerulonephritis, severe hypertension (210/120), uremia.	180	End-to-end anastomosis profunda femoris artery with renal artery. End-to-side renal vein into common femoral vein. Pedicle graft swung. Right leg. Kidney encased in polyethylene bag.	None	None	200 mgm.—24° 50 mgm./D for 62 D	Penicillin Streptomycin Aureomycin Terramycin® from 29th to 145th day	Tremendous hematoma developed under flap. This was evacuated on 8th p.o. day.	None	Diuresis began 19th day and function reached maximum on 37th day. Function persisted for 5½ months.

duct by tumor. A communication was established *via* a polyethylene tube between the ureter and the subarachnoid space in one case, and between the ureter and ventricle in the other, to relieve the increased intracranial pressure. This operation, which was developed and performed by Dr. Donald Matson (89), necessitates the removal of one kidney.

The recipients were all patients in irreversible terminal uremia on the basis of chronic pyelonephritis in five cases, chronic glomerulonephritis in two cases, polycystic kidney disease in one case, and periarteritis nodosa in one case. The transplanted kidneys came from donors of the same blood type as the recipients in seven cases, and different blood types in two cases.

When hemodialysis was necessary it was carried out with a modification of the Kolff artificial kidney (99).

One patient (F. A.), a 37-year-old man with chronic pyelonephritis and severe uremia, was referred to the Peter Bent Brigham Hospital where he was twice dialyzed on the artificial kidney during a 14-day hospitalization. He was then transferred to an outside hospital where a donor kidney was available. A transplant was performed by Dr. James V. Scola.[2] The left kidney and spleen of the recipient were removed and anastomoses were made end-to-end between the splenic artery and the renal artery, and between the two renal veins. The pelvis of the transplant was anastomosed to the ureter of the recipient, and a T-tube was brought out from the ureter through a stab wound. The patient was readmitted to the Peter Bent Brigham Hospital on the 3rd day, and was followed by us until his death on the 37th day.

Sodium and potassium were determined with the Beckman flame photometer; inulin by the method of Schreiner (111); para-amino-hippurate by the method of Finkelstein, Aliminosa, and Smith (44); urea by the method of Archibald (2); chloride by the method of Schales and Schales (109), and Asper, Schales and Schales (3); urine creatinine by the method of Bonsnes and Taussky (14); phosphorus by the method of Fiske and Subbarow (45); ammonia by the method of Folin and Bell (47); carbon dioxide by the method of Van Slyke and Neill (129); and urinary protein by the method of Chinard (27). Urinary sediment was evaluated by the semiquantitative method of Addis (1).

RESULTS

A summary of the results in the cases reported here is shown in Table I. A detailed report of the cases follows in their chronological order.

Case I—F. A., male, age 37, No. 8B438

Diagnosis: Chronic pyelonephritis, hypertension, uremia.

The patient was referred to the Peter Bent Brigham Hospital from the Springfield Hospital, Springfield, Massachusetts, on 3-20-51 by Dr. L. H. Doolittle. A diagnosis of acute nephritis had been made at the Springfield Hospital 18 years previously. In 1950 he was again admitted there and studies were made which led to a diagnosis of chronic pyelonephritis. He had known hypertension for two years. For three months before admission, he had complained of nausea, vomiting, drowsiness, and increasing weakness.

Physical examination showed a chronically ill, semicomatose man complaining bitterly of back pain. Optic fundi showed narrowing of the arterioles with an occasional fresh punctate hemorrhage. Lungs were clear. The heart was slightly enlarged to percussion, and there was an apical presystolic gallop. Blood pressure was 170/90. There was bilateral costo-vertebral tenderness.

Laboratory data: The urine specific gravity was 1010 with 4+ proteinuria, 5-10 red cells, and a few white blood cells in the unspun sediment. The hematocrit was 24, white blood count 6,900, blood urea nitrogen 296 mgm. per cent, carbon dioxide combining power 10 mM. per L., serum sodium 132 mEq. per L., serum potassium 5.2 mEq. per L. Urine culture grew enterococci and *E. coli*. A chest film showed the heart to be at the upper limits of normal, the contours suggesting left ventricular hypertrophy. The vascular markings were increased.

In spite of 5 days of conservative therapy, there was no improvement in the clinical status. On the 6th hospital day, dialysis with the artificial kidney was performed with good chemical result but little clinical change. The patient was again dialyzed on the 10th hospital day, and following this procedure, there was definite marked clinical improvement.

On 3-30-51, the day of the last dialysis, the patient was transferred to the Springfield Hospital. The following day a renal homograft was performed by Dr. James V. Scola. The donor was a patient with carcinoma of the lower end of the ureter whose kidney was removed by Dr. L. H. Doolittle at the same time that exposure was being obtained in the recipient. Both patients were blood type A, Rh+. A left nephrectomy and splenectomy were performed, and the splenic artery was anastomosed to the renal artery of the homograft, and the renal vein to renal vein. The recipient's ureter was anastomosed to the pelvis of the transplant, and a T-tube was placed in the ureter and brought to the outside. The period of ischemia of the transplanted kidney was 70 minutes. The patient tolerated the procedure well, and on the 4th day he was readmitted to the Peter Bent Brigham Hospital. Following operation, the urine volume had decreased, although the clinical condition on readmission was generally better than prior to operation. The patient received seven blood transfusions on the operative day.

Physical examination on the *second admission* was much as before, with the exception that breath sounds were absent and tactile fremitus reduced over the left lower lung field. A marked pericardial friction rub was present. The abdomen was now distended and tympanitic, although peristalsis was present. The hematocrit was 32, white blood count 22,400, blood urea nitrogen 101 mgm. per cent, serum carbon dioxide combining

[2] Springfield Hospital, Springfield, Massachusetts.

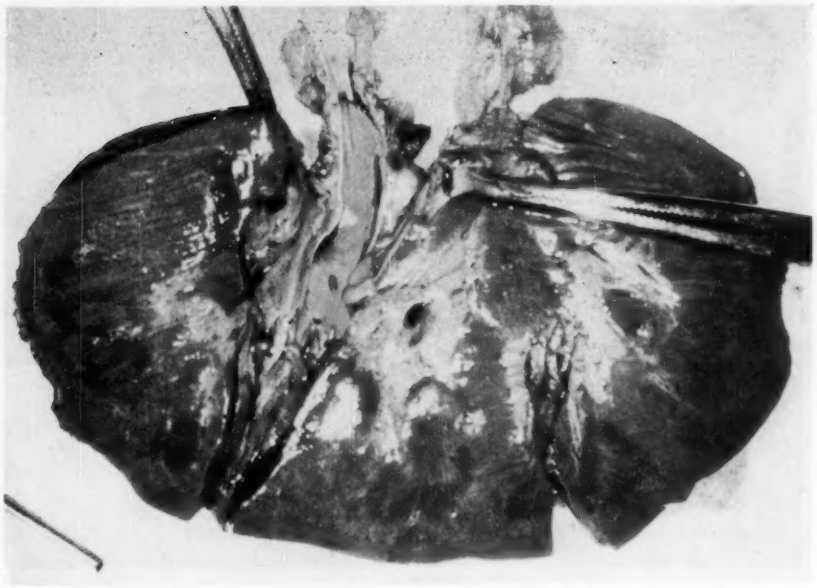


FIG. 2. F. A., CASE NO. 1—THE APPEARANCE OF THE CUT SURFACE OF THE RENAL HOMOGRAFT

Grossly, the transplant appears normal, except for a slight mottling. Both anastomoses are patent.

power 15 mM. per L., sodium 140 mEq. per L., potassium 6.9 mEq. per L. X-ray of the chest showed a small area of consolidation at the left base, and the sputum cultured *Pseudomonas aeruginosa* which was almost completely resistant to all antibiotics.

He was heparinized for the first 2 days following operation, and subsequently received 200 mgm. of heparin a day from the 5th to the 9th day. He was given 20 mgm. per day of ACTH intramuscularly (I-M) [4] for 3 days. On the 4th day he received 65 mgm. I-M, and from the 5th through the 11th day he received 25 mgm. per day intravenously (I-V) [4] or 100 mgm. I-M. It was tapered off from the 11th to 13th day. No cortisone was given. He received 100 mgm. of testosterone propionate on the 15th day, and 50 mgm. per day thereafter from the 16th through the 37th day.

On the 9th day, because of deterioration in the clinical condition, hemodialysis was performed with good clinical response. The abdominal distention, however, continued to be a major problem. Hemodialysis was performed again on the 18th, the 26th, and the 32nd days with deterioration of the clinical state between each procedure. The urine output varied between 300 and 100 ml., of which varying amounts came from the ureteral T-tube of the homograft. It was difficult to evaluate what percentage of the T-tube drainage was reflux from the bladder urine. Indigo carmine was injected once and PSP twice, but neither of these dyes appeared in bladder or T-tube urine.

On the 15th hospital day, a friction rub appeared in the right axilla. On the 23rd day, because of separation and infection of the abdominal wound, a debridement and secondary closure were carried out. On the 27th day a retrograde injection of contrast media into the T-tube catheter showed what appeared to be a small drainage cavity resulting in some break in the continuity of the suture line near the renal pelvis of the homograft. On the 34th day a left thoracentesis yielded 620 ml. of apple-jelly colored fluid. The patient now appeared brighter. On the 37th day he complained of chest pain, and shortly after an intercostal block for this pain, the patient gasped suddenly and expired.

Post-mortem examination showed chronic pyelonephritis of the right kidney, perinephric abscess about the left homograft, empyema of the right pleural cavity, left pulmonary atelectasis, and left ventricular hypertrophy. Gross examination of the homograft showed it to be slightly enlarged and somewhat mottled. Except, however, for the perinephric abscess, the gross appearance was not markedly abnormal (see Figure 2). Microscopic examination showed a dense avascular connective tissue sheath surrounding the capsule of the transplanted kidney. This reaction appeared to be mostly extra-capsular (see Figure 3). The glomeruli appeared

[4] Hereafter I-M will be used as an abbreviation for intramuscularly, and I-V for intravenously.

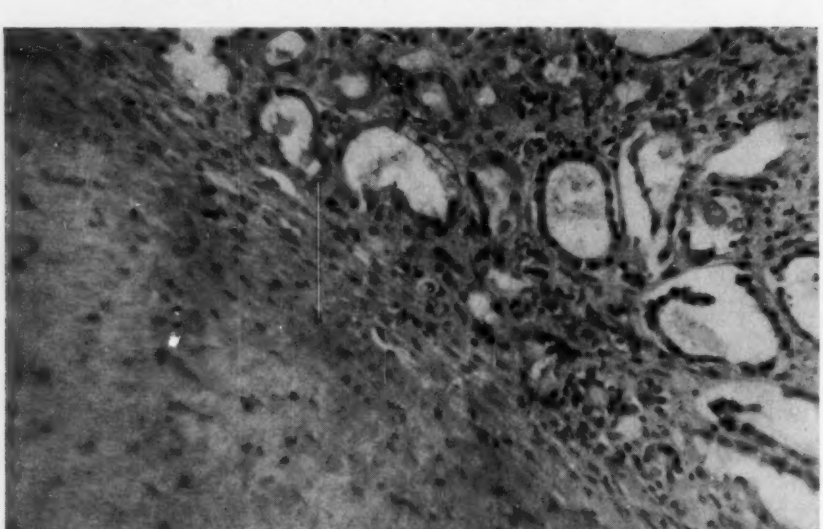


FIG. 3. F. A., CASE NO. 1—A DENSE, AVASCULAR CONNECTIVE TISSUE CAPSULE SURROUNDS THE TRANSPLANTED KIDNEY (H & E × 250)

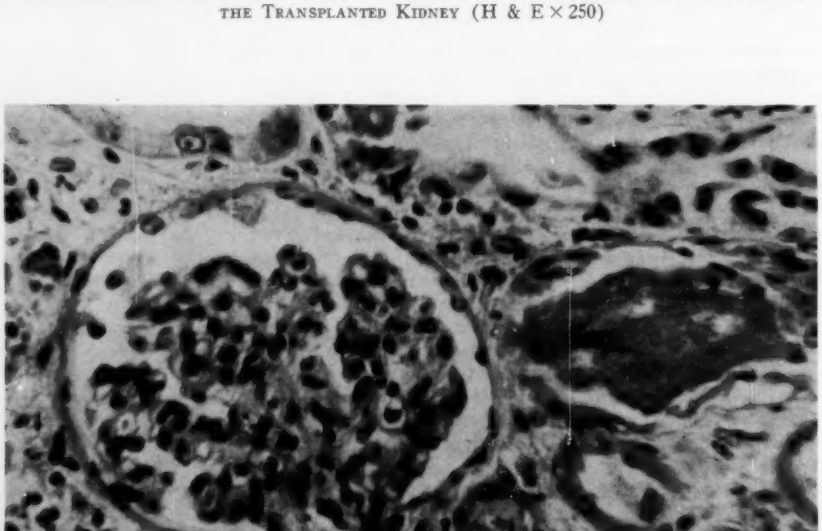


FIG. 4. F. A., CASE NO. 1—A GLOMERULUS IS RELATIVELY BLOODLESS, BUT SHOWS NO APPRECIABLE INFLAMMATION OR PROLIFERATION—THERE IS DEGENERATION OF THE EPITHELIUM OF ADJACENT TUBULES, AND BASOPHILIC CASTS ARE PRESENT (H & E × 500)

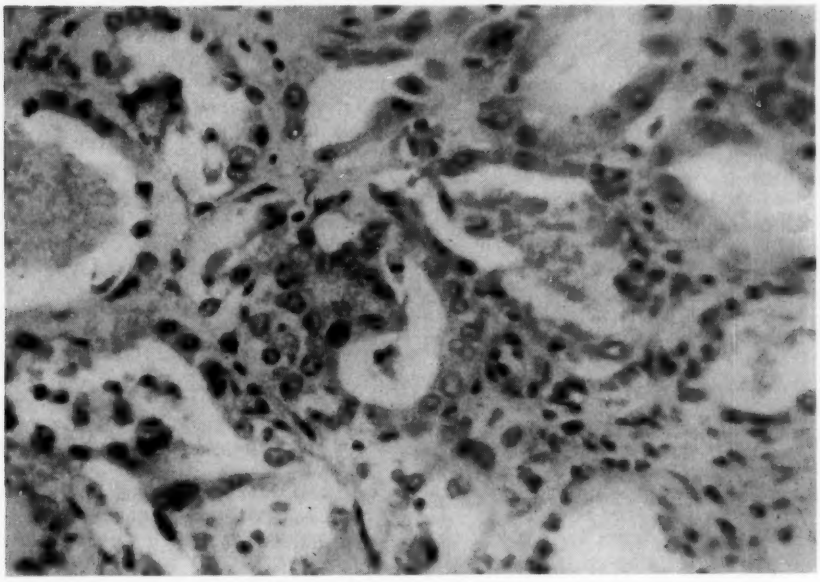


FIG. 5. F. A., CASE NO. 1—THE EPITHELIUM OF THE CONVOLUTED TUBULE IN THE CENTER OF THE ILLUSTRATION IS HEAPED UP AND NUCLEI ARE CLOSELY APPROXIMATED, SUGGESTING FOCAL REGENERATIVE PROLIFERATION—IN NEARBY TUBULES, DEGENERATIVE CHANGES PERSIST (H & E × 500)

relatively bloodless, but there was no appreciable inflammation or increased cellularity (see Figure 4). The tubules showed a moderate amount of degeneration, which was most prominent in the proximal tubules and in the loops of Henle. Calcium oxalate crystals were present in the lumen of the tubules. There was also some regeneration of tubular epithelium which was most noticeable in the distal tubules (see Figure 5). The histologic picture was not unlike that of a severe acute ischemic nephrosis ("lower nephron nephrosis") in a repairing state. There was very little interstitial infiltration.[5]

Case II—M. P., female, age 30, No. 6B376

Diagnosis: Chronic pyelonephritis, hypertension, uremia.

This 30-year-old white female was admitted to the Peter Bent Brigham Hospital for the second time on 3-14-51. On a previous admission, 13 months before, the diagnosis of hypertensive vascular disease, pyelonephritis, and uremia had been made. The hospital stay revealed positive cultures from each ureter, proteinuria, hypertension without cardia enlargement, and a blood urea nitrogen of 40 mgm. per cent. The present admission was occasioned by severe nausea and vomiting following an upper respiratory infection. In an outside hospital, in spite of therapy, this had progressed to semi-coma accompanied by twitching and convulsions.

Physical examination: On admission the patient was a critically ill, dehydrated, semi-comatose young woman. Blood pressure was 158/100. The optic fundi were unremarkable except for moderate arterio-venous nicking. The heart was now enlarged by x-ray, and there was a presystolic gallop with a grade II apical systolic murmur. There was no venous distension, and no peripheral edema. A few dry rales were heard at the left base. The reflexes were markedly hypoactive.

Laboratory data: The blood urea nitrogen was 113 mgm. per cent, carbon dioxide combining power 14.1 mM. per L., serum chloride 86 mEq. per L., sodium 133 mEq. per L., potassium 4.3 mEq. per L., and phosphate 4.3 mEq. per L. The hematocrit was 19, and the white blood count was 5,300 with a normal differential.

Hospital course: The patient showed very little improvement after 8 days of conservative therapy, and on 3-22-51 hemodialysis with the artificial kidney was performed. There was a temporary improvement following this. However, on 4-14-51 the patient again had a generalized convulsion, following which she was maintained on Dilantin® without further convulsive episodes. By

[5] For the description of the pathology of the homotransplants in Cases 1 through 8 we are indebted to Dr. Clinton Van Zandt Hawn, formerly Pathologist-in-Chief of the Peter Bent Brigham Hospital, and at present Pathologist-in-Chief of the Mary Imogene Bassett Hospital, Cooperstown, N. Y. For the description in Case No. 9 we are indebted to Dr. Gustave Dammin, Pathologist-in-Chief of the Peter Bent Brigham Hospital.

4-20-51 the blood urea nitrogen had risen to 210 mgm. per cent with a serum potassium of 7.9 mEq. per L. On 4-23-51 a renal homograft was transplanted into the right thigh, employing a kidney from a patient who had died during a surgical procedure for hypertensive vascular disease. The donor's blood type was O, Rh +, while that of the recipient was A, Rh +, thus presenting compatibility in the major grouping but not in the minor. The period of time which elapsed between the death of the donor and the re-establishment of blood flow through the transplant in the host was 150 minutes. (In subsequent cases this interval will be spoken of as the period of ischemia of the kidney. In cases in which the donor was living, the beginning of the interval will be reckoned from the time when the renal vessels were clamped.)

The kidney was perfused with normal saline solution as soon as it was removed from the cadaver, because of the blood group incompatibility. End-to-end anastomoses were made between the profunda femoris artery and the renal artery, and between the superficial femoral vein and renal vein. The bulldog clamp was removed from the femoral vein and heparin was inserted through a No. 26 needle into the renal artery. The arterial clamp was now removed, and with the re-establishment of the circulation, the kidney became pink. There are two details worth noting at this point: 1) By mistake 125 mgm. of heparin, instead of 25 mgm., was injected into the renal artery, and thus into the general circulation—this was followed shortly by oozing from the wound edges; and 2) this kidney and the kidney in Case No. 6 were not quite as vigorously or quickly distended with blood when the clamps were removed as the other transplants were. Both these kidneys, unlike the others, came from hypertensive donors, whose blood types were different from that of the recipient, and both kidneys were perfused before being anastomosed. One or all of these three factors may have had some bearing on the apparently initially somewhat diminished blood flow of these kidneys compared to the other grafts.

A pocket was fashioned between the fascia of the adductor muscles and the subcutaneous tissue, and the subcutaneous tissue and skin were closed over the kidney. The ureter was brought out through a stab wound.

For 48 hours the patient was given heparin, 40 mgm. every 6 hours I-M, and ACTH, 20 mgm. every 8 hours I-V. She was then given 225 mgm. of cortisone and 50 mgm. of testosterone daily for 7 days. Hemodialysis was again performed on the 2nd day because of her poor clinical condition, and she improved markedly following this. A large hematoma developed beneath the skin flaps, and by the 3rd day the skin overlying the transplant had become shiny and tense, and the ureter was necrotic. It was decided to re-explore the transplant. There was a large hematoma around the kidney. The kidney was a normal pink color, and there was a good blood flow through it. The capsule bled freely, and it was therefore replaced and a relaxing incision was made in the skin laterally.

Sepsis developed in the wound. A biopsy of the kidney on the 13th day showed infarction of the kidney, and it was removed 4 days later. The renal vessels were thrombosed and massive infarction of the kidney had occurred. Six days later the infection in the wound eroded through the superficial femoral artery, and a severe hemorrhage occurred. The patient was reoperated and it was found that an eroded area in the artery 2 cm. long and 0.5 cm. wide had given way. It was impossible to suture the vessel because the wall was necrotic. A lucite tube was placed in the vessel, therefore, and tied in with nylon rings after the method described by Hufnagel and Gillespie (57). A skin graft was laid over the blood vessel. During the next 2 days the sensation, movement, and temperature of the leg were normal. Good pulses could be felt in the foot. At this time the patient had an episode of pulmonary and cerebral edema and died suddenly. At post-mortem the lucite tube was patent, and the femoral artery appeared viable on either side of the nylon rings which held the tube in place.

Gross and microscopic examination of the homograft in this case revealed total infarction of the tissues, the renal structure being so distorted that no other changes were discernible.

Case III—F. Z., female, age 43, No. 5A157

Diagnosis: Chronic pyelonephritis, hypertension, arteriosclerosis, uremia.

This was the sixth admission of this patient with chronic progressive uremia who had been treated on two previous occasions by the artificial kidney with satisfactory results. Her blood urea nitrogen prior to dialysis had been as high as 188 mgm. per cent. She was admitted to the hospital on this occasion specifically for a renal homograft, having been discharged one week previously, following dialysis.

Physical examination: The patient was a well developed woman with dry scaling skin and generalized pallor. The blood pressure was 165/90. There was slight sclerosis of the retinal vessels. The lung fields were clear, and the heart was enlarged to percussion with a protodiastolic gallop and a course systolic bruit at the fourth interspace. Chest x-ray showed hilar congestion and marked cardiac enlargement. The liver was enlarged three finger breadths below the right costal margin and was slightly tender. There was minimal presacral and pretibial edema.

Laboratory data: The urine showed 2 + proteinuria, and 10–20 white cells with rare granular casts in the spun sediment. The blood urea nitrogen was 96 mgm. per cent, serum carbon dioxide combining power 11.6 mM. per L., sodium 130 mEq. per L., and potassium 6.8 mEq. per L.

On 4-24-51, the day of admission, a renal homograft was done. The donor was a patient with severe mitral stenosis who died during a valvuloplasty. Her urine contained from 1 + to 4 + albumen on many occasions, but her blood urea nitrogen was within the normal range.

TABLE II

Case No. 3—A comparison between the sodium, potassium, creatinine, and urea nitrogen content of the bladder urine and the transplant urine. The collection of urine from the transplanted kidney was not complete

F. Z.—5-13-51—19th day. BUN: 148

	24-hr. Vol.	Na *mEq./L.*	K *mEq./L.*	Creatinine *mgm. %*	Creatinine *mgm./24°*	Urea N *mgm. %*	Urea N *gram/24°*
Bladder urine	580	16.2	35	44	225	832	4.8
Transplant urine	600*	3.5	47	52	312+	1,023	6.1+

* Not complete.

Examination of her other kidney showed multiple small infarcts—apparently caused by emboli. Both donor and recipient were blood group B, Rh+, and their bloods were cross-matched and found to be compatible. The period of ischemia was 135 minutes. The kidney was transplanted into the right thigh by means of an end-to-end anastomosis of the profunda femoris artery with the renal artery, and an end-to-side anastomosis of the renal vein into the common femoral vein. The kidney was not perfused. There was marked sclerosis of the recipient's vessels, and some sutures were taken through arterio-sclerotic plaques. Nonetheless the kidney became distended with blood as soon as the clamps were removed, and was a normal pink color. The kidney was placed in a pocket created between the fascia overlying the adductor muscles and the subcutaneous fat. The subcutaneous tissue and skin were closed over the kidney, and there was a moderate amount of tension on the skin. The patient received 25 mgm. of ACTH every 6 hours for 24 hours, and was then given 225 to 300 mgm. of cortisone daily for 18 days, at which time it was reduced to 100 mgm. daily and ACTH, 80 mgm. a day, was begun again. The cortisone was stopped after 25 days, and the ACTH was gradually tapered off after 31 days. She was given 40 mgm. of heparin every 6 hours for 14 days, and 50 mgm. of testosterone daily for 29 days, at which time it was tapered off and stopped on the 34th day. Penicillin and Purodigin® were also given.

During the first week after the transplantation, necrosis of the skin overlying the kidney began to develop because of edema in the wound and tension on the skin flaps. The ureter was not yet involved in this process, but it was edematous and it ultimately became markedly swollen and pale. After 8½ days of anuria the trans-

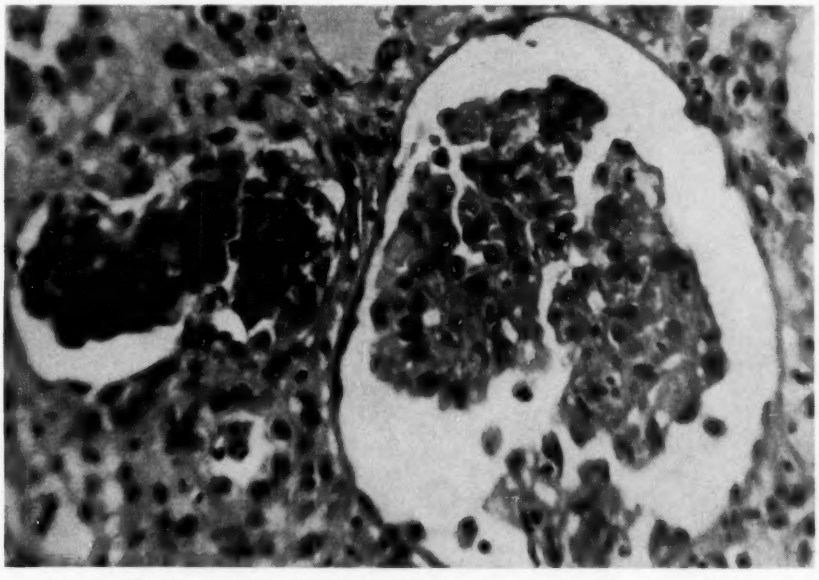


FIG. 6. F. Z., CASE NO. 3—BIOPSY OF THE HOMOGRAFT TAKEN ON THE 70th DAY

The glomerular tuft is relatively bloodless, but shows no appreciable exudative or proliferative response. An adjacent tubule is denuded of epithelium and contains an irregularly laminated calcific mass. (H & E × 500)

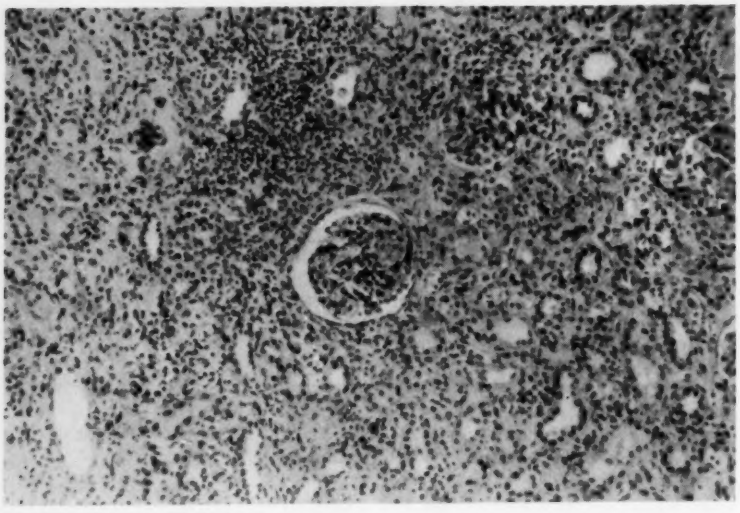


FIG. 7. F. Z., CASE NO. 3—BIOPSY OF THE HOMOGRAFT TAKEN ON THE 70th DAY

There is marked infection, with round cell and polymorphonuclear leucocytic infiltration of the interstitial spaces. There is no evidence of acute or chronic glomerulonephritis. The glomerulus in the center of the picture is somewhat ischemic, but otherwise appears surprisingly normal. (H & E × 150)

planted kidney began to excrete urine. The urine was collected by letting the tip of the ureter hang down into a polyethylene sac which led through tubing to a bottle. These collections were somewhat inaccurate, at first because the ureter would sometimes pull out of the sac when the patient turned in bed, and later because as progressive slough of the ureter occurred, there was leakage from the wound. On the 14th day, 6 ml. of indigo carmine was injected intravenously. Small quantities of dye were noted to appear in the urine from the transplanted kidney, but none was seen in the bladder urine. By the 18th day the transplant had reached an output of more than 600 ml. per day. A phenolsulfonphthalein excretion (PSP) test was performed on this day and showed an output of 10 per cent of the dye in 2 hours in the urine from the transplanted kidney and less than 5 per cent in the bladder urine. An injection of aminophyllin intravenously produced a marked transient increase in urinary output from the transplanted kidney. A comparison between the bladder and transplant urine is shown in Table II.

The slough of skin over the kidney continued to increase in size, and after the 22nd day it was no longer possible to obtain accurate urine collections.

On the 24th day the patient was re-operated and a polyethylene tube was placed in the wound and led to a gravity drain bottle, and wound suction was started over the dressings in an attempt to get more complete collections and keep the patient's leg drier. About 500 ml. per day was collected by these routes. After 6 days the slough had spread so much the tube had to be removed.

PSP tests were repeated on the 22nd and 31st days, and dye was seen to appear within 10 minutes each time. No quantitative tests were possible. Urinary output began to diminish from the 33rd day on, although by the 53rd day the kidney was still excreting an estimated 200 to 300 ml. per day. On the 57th day the patient was dialyzed because of deterioration of her clinical state.

As the slough spread, the kidney finally became almost completely exposed. The wound was debrided and granulation tissue grew over the transplant. The cortex still bled vigorously when cut. On the 70th day a biopsy of the kidney was performed. The kidney parenchyma bled freely during the biopsy. Microscopically the biopsy showed the glomeruli to be relatively bloodless, but without any specific exudative or proliferative response (see Figure 6). There was edema and polymorphonuclear and round cell infiltration of the interstitial spaces (see Figure 7). The predominate picture was that of infection—pyelonephritis and interstitial nephritis—associated with a conspicuous calcific degeneration of the tubular epithelium. There were pigment casts and calcific masses in the tubular lumina (see Figure 8). Thrombosis of some of the smaller vessels could be seen, associated with focal infarcts.

On the 73rd day a retrograde pyelogram was done on the homograft. The renal pelvis and calyces appeared fairly normal. The urinary output fell off almost to nothing. The entire transplant became necrotic and

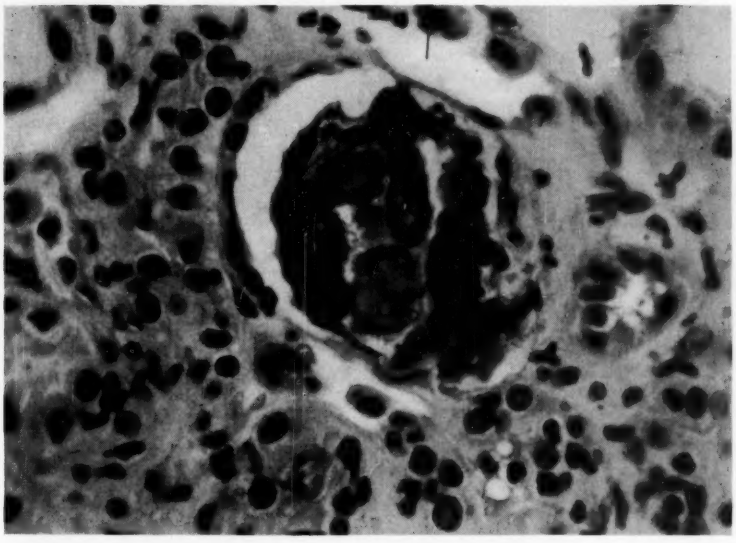


FIG. 8. F. Z., CASE NO. 3—BIOPSY OF THE HOMOGRAFT TAKEN ON THE 70th DAY
There are laminated basophilic casts in the tubular lumen. (H & E × 400)

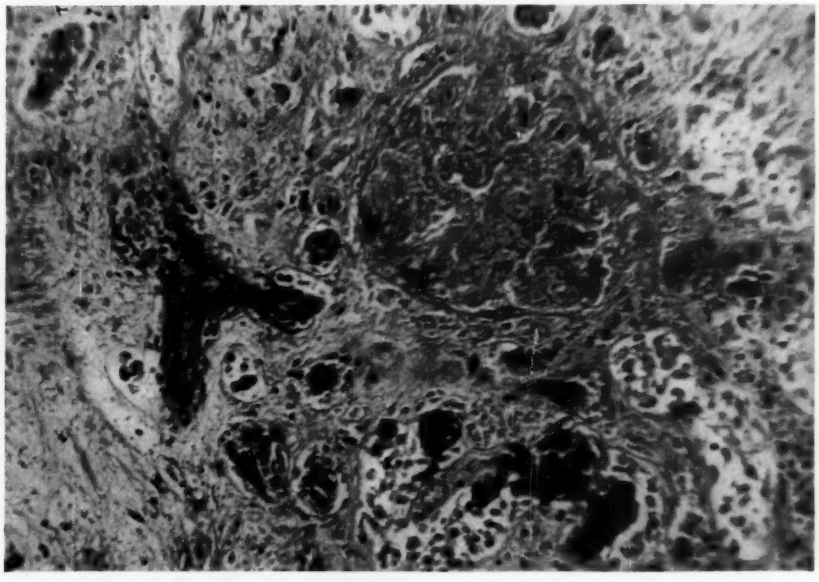


FIG. 9. F. Z., CASE NO. 3—THE MICROSCOPIC APPEARANCE OF THE TRANSPLANT AT POST-MORTEM

Infarction and necrosis of the kidney occurred. The glomerular infarction is not associated with appreciable evidence of pre-existing inflammatory or proliferative changes. Nearby blood vessels are thrombosed. Many tubules contain densely basophilic masses. (H & E × 250)

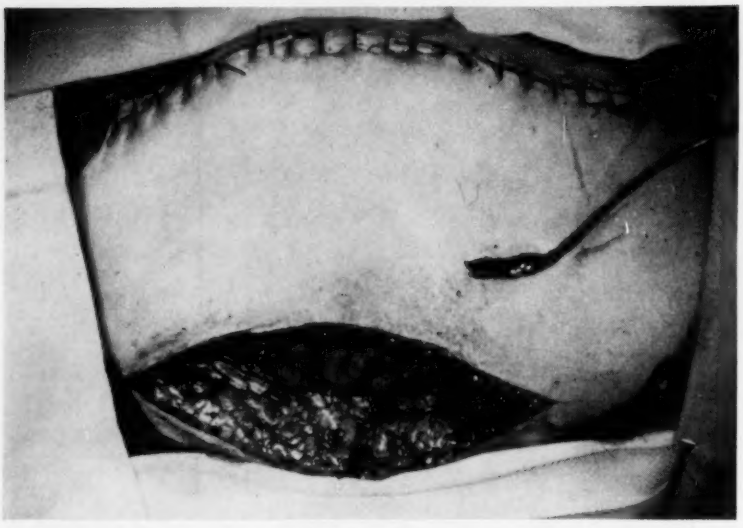


FIG. 10. B. B., CASE NO. 4—VIEW OF THE RIGHT THIGH ON THE DAY OF OPERATION, SHOWING METHOD OF SLIDING DOUBLE-ENDED FLAP OF SKIN AND SUBCUTANEOUS TISSUE OVER THE KIDNEY TO ELIMINATE TENSION ON THE SKIN SUTURE LINE

The ureter can be seen projecting through a stab wound. A catheter in the ureter leads to a drainage bottle. A denuded area is left over the fascia lata laterally.

thrombosed, and the patient went steadily downhill and died on the 99th day.

Post-mortem examination was limited to the homograft, and showed extensive autolysis and vascular thrombosis (see Figure 9). Marked evidence of infection, with colonies of bacteria, and extensive infarction, were seen. Again the dominant inflammatory cell was the polymorphonuclear leucocyte, and there was no evidence of acute arteritis.

Case IV—B. B., female, age 52, No. 7B634

Diagnosis: Polycystic kidney disease, pyelonephritis, hypertension, uremia.

This was the third admission of a 52-year-old housewife with known polycystic kidney disease. She had been admitted to this hospital for the first time 7 months before the present admission for drowsiness, vomiting, and oliguria. Good symptomatic improvement was obtained by dialysis with the artificial kidney on two occasions. She was again admitted 7 weeks before the present admission, being discharged 4 weeks later. Her present admission on 5-24-51 was in preparation for a renal homotransplant.

Physical examination: On this occasion, examination revealed a well developed white female in no apparent distress. The skin was dry and rough with spotty café-au-lait pigmentation. Blood pressure was 170/100. The arterioles of the fundi showed some irregularity and slight A-V nicking. The heart was enlarged to percussion with a grade I apical systolic murmur, and normal sinus rhythm. The lungs were clear. The kidneys were enlarged and easily palpable. There was no peripheral edema.

Laboratory data: The urine was clear, with a specific gravity of 1.009, and 2+ proteinuria. The sediment showed no red blood cells or casts, but 3 to 4 white blood cells per high power field. Hematocrit was 33, white blood count 6,900, blood urea nitrogen 78 mgm. per cent, serum total protein 6.3 gm. per cent, carbon dioxide combining power 18 mM. per L., chloride 103 mEq. per L.

On 5-26-51 a kidney was transplanted into the right thigh. The donor was a patient with tetralogy of Fallot who died 4 days after an operation to widen the pulmonary valve. Both donor and recipient were blood type B, Rh +, and their bloods were cross-matched and found to be compatible. An end-to-end anastomosis was again made between the profunda femoris artery and the renal artery, and an end-to-side anastomosis was performed between the renal vein and the common femoral vein. The kidney was not perfused. The period of ischemia was 200 minutes.[6] An injection of 25 mgm. of heparin was made into the renal artery with a No. 26 needle. Circulation through the kidney appeared excellent, and the ureter, which was left about 5 inches long,

[6] This long period of ischemia was occasioned by a delay in obtaining the donor kidney.

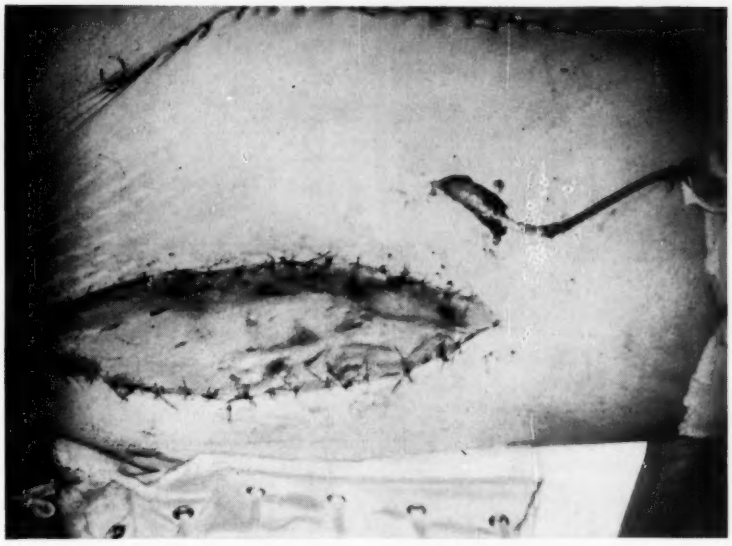
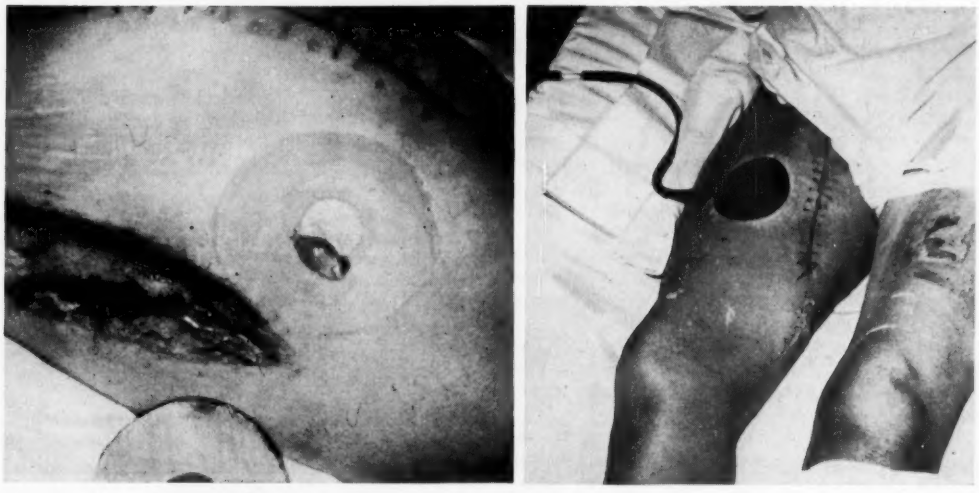


FIG. 11. B. B., CASE NO. 4—VIEW OF THE RIGHT THIGH ON THE DAY OF OPERATION, SHOWING HOW A SKIN GRAFT WAS PLACED TO COVER THE DEFECT IN LATERAL PORTION OF THE THIGH



A B

FIG. 12. B. B., CASE NO. 4—VIEW OF THE RIGHT THIGH ON THE 18th DAY, SHOWING THE METHOD OF COLLECTING THE URINE

At the left the ureter may be seen protruding above the skin (A). On the right, the ureterostomy cup has been glued in place (B).

TABLE III

B. B. Case No. 4—A comparison between transplant and bladder urine at representative periods throughout the patient's course

	Day	24-hr. Volume		Specific gravity		Creatinine *mgm. %*		Creatinine *mgm./24 hr.*		Urea nitrogen *mgm. %*		Urea nitrogen *gm./24 hr.*		Sodium *mEq./L.*		Potassium *mEq./L.*		Chloride *mEq./L.*	
		B*	T*	B	T	B	T	B	T	B	T	B	T	B	T	B	T	B	T
Period of maximal function	14	1,600	1,100	1.010	1.011					293	390	4.7	4.3					40	43
	16	1,575	1,560	1.011	1.020	23	34	360	534										
	17	875	900	1.007	1.010	17	35	148	314	233	450	2.0	4.1						
	18†		2,826																
Period of declining function	33	2,500	400			12	56	298	223	132	280	3.3	1.1					59	17
	37	2,900	565			17	48	479	273	198	417	5.8	2.4					64	22
	40	2,100	775	1.004	1.006	18	45	378	349	300	571	6.3	4.4	53	15	21	52	53	17
	44	1,860	745	1.002	1.006					309	352	5.8	2.6	51	9	23	48	43	21
	47	1,800	450	1.010	1.005	18	49	326	221	290	550	5.2	2.5	54	12	17	42	53	29
	54	1,500	700	1.003	1.004	18	47	270	329	330	487	5.0	3.4	55	17	18	42	45	37
	58	1,400	350	1.003	1.005	20	64	280	224	381	552	5.3	1.9					49	42

* B: Bladder urine.
T: Transplant urine.
† No creatinine or urea nitrogen determinations were done on the days when the transplant function was probably at its peak (days 18 through 26).

was well vascularized, and active peristaltic waves passed along it.

A pocket was again created between the adductor fascia and the subcutaneous fat, but this time a large 15 by 30 cm. double-ended pedicle flap of skin and subcutaneous fat was raised laterally off the anterior and lateral deep fascia. This pedicle was used to cover the kidney. The fascia lata, which was left bare by moving this flap over, was covered with a split thickness skin graft taken from the other leg. The ureter was brought out through a small incision in the pedicle, and allowed to project about 1 inch past the skin margin. A catheter was left in the ureter for 2 weeks. These relationships are shown in Figures 10 and 11. The skin was closed without tension.

The patient was treated with heparin, 20 mgm. I-M every 6 hours for 24 days. Cortisone 100 mgm. I-M every 8 hours was given for 1 week, then 50 mgm. every 8 hours for 1 week. It was then gradually tapered off over the next 4 days, being stopped on the 18th day. ACTH, 100 mgm. per day, was given from the 8th to 21st day, and it was then tapered off over the next 3 days. On the 29th day cortisone, 150 mgm. a day, was again resumed and continued until the 45th day. Testosterone propionate, 50 mgm. a day, was given from the 1st to the 25th day, and from the 66th to the 90th day. Penicillin was given for the first 30 days, following which various antibiotics were administered, including Chloromycetin®, Aureomycin®, and Terramycin®.

Following the operation, the tip of the ureter protruding through the skin ureterostomy appeared pink and healthy. It was necessary on the 3rd day to ligate a small bleeding artery on the tip of the ureter.

The wounds healed well. For the first 11 days after the operation the transplant was essentially anuric, secreting only a few ml. of clear urine. On the 12th day, however, urine began to flow in gradually increasing amounts reaching a maximum 24-hour output of 2826 ml. on the 18th day. The urine was collected by fastening a regular ureterostomy cup over the ureter with skin glue, and leading the outflow to a bottle beside the patient's bed, or to a bag strapped to her leg when she was up (see Figure 12).

The specific gravity of the urine from the transplant varied between 1.008 and 1.020. The sediment contained 2 to 3 white blood cells and 3 to 4 red blood cells per high power field, as contrasted with 25 to 30 white blood cells and 0 to 1 red blood cells per high power field for the bladder urine. The protein varied from 1 + to 2 +, while the bladder urine ran 1 + to 3 +. Urine sugar was negative. Both bladder and transplant urine contained many bacteria, and cultures showed various coliform organisms which were resistant to all antibiotics. A comparison between the transplant urine and bladder urine at various times in the post-operative period is shown in Table III. It may be seen from this chart that during

TABLE IV

B. B. Case No. 4—Kidney function studies done simultaneously on the transplanted kidney and the recipient's own kidneys

The 18th day	Urea clearance *ml./min.*	Creatinine clearance *ml./min.*	PSP *%/2 hrs.*
Both host kidneys	2.5	5.8	0
Transplant	6.4	18.5	22
Transplant × 2	12.8	37.0	44
Normal	71.0	148.0	75

The 21st day	Inulin clearance *ml./min.*	Plasma flow *ml./min.*
Both host kidneys	2.1	11
Transplant	14.4	112
Transplant × 2	28.8	224
Normal	126.0	620

TABLE V

A comparison of the urinary output of the transplanted kidney with the blood urea nitrogen and serum potassium concentrations of the recipient

	Post-op. day	24-Hour urine volume from the transplant	BUN	Serum K
Period of maximal function	13	400+	140	4.3
	14	1,100		
	15	1,125		
	16	1,560		
	17	900	94	4.3
	18	2,826		
	19	1,500+		
	20	lost	65	2.8
	21	1,350		
	22	1,660		
	23	lost		
	24	1,100		
	25	1,050		
	26	950	41	3.2
Period of declining function	34	435	65	4.3
	41	780	130	6.8
	47	450+	136	5.8
	51	650	122	5.6
	65	225	154	
	66	hemodialysis performed		

the period of maximal function the transplant urine continued to show greater concentrations of urine urea nitrogen, creatinine, and potassium, and lower concentrations of sodium and chloride than the bladder urine.

Renal function studies were carried out simultaneously on the homograft and the patient's own kidneys. The results of one of these studies are shown in Table IV. During the period of maximal function of the transplant, between the 14th to 28th day, the patient's blood urea nitrogen and potassium steadily dropped, and the patient became symptom-free for the first time in two years. As the function of the transplant dropped off again, the blood urea nitrogen and potassium rose, and it was necessary to dialyze the patient on the 66th day (see Table V). It was of interest that the volume of the bladder urine decreased as the transplant diuresed, and increased again as the volume of the transplant fell off.

The patient became febrile on the 25th day, and on the 29th day she had a chill with a temperature to 104°. This was accompanied by tenderness and heat over the transplanted kidney, and some pain in the leg. It appeared that the patient had developed a pyelitis of this kidney. Chloromycetin® therapy was re-instituted, and cortisone was given, as well, because of the feeling that the "immune response" might be playing some part in this reaction. There was a gradual fall in temperature over the next 5 days, accompanied, however, by a decrease in the output of the homotransplant. It is interesting that after this acute episode had subsided somewhat the transplant was again able to increase its urinary volume and urea nitrogen output temporarily.

The temperature again rose to 103° on the 41st day, and thereafter, in spite of antibiotic therapy, the daily

FIG. 13. B. B., CASE NO. 4—THE 83RD DAY

A retrograde pyelogram of the transplanted kidney shows no evidence of obstruction or distortion of the calyces, pelvis, or ureter.

rises were accompanied by a deterioration in the clinical condition. The spiking fever persisted until the 84th hospital day, at which time there was an 8-day period when the patient was afebrile. Output from the homograft had diminished at this time to 50 to 75 ml. a day. On the 92nd day she again developed a swinging, septic type of fever, and the clinical condition again deteriorated. Hemodialysis was carried out on the 98th day and again on the 100th day in order to prepare her for exploration of the homograft site. The urine from the transplant now was scanty with a greenish sediment. Retrograde pyelograms of the homograft made on the 48th and 83rd days demonstrated no evidence of obstruction or deformity. The pyelogram obtained on the 83rd day is shown in Figure 13.

The ureter continued to remain well vascularized. The mucosa grew out of the end of the ureter and down the outside, until it ultimately reached the skin. As the kidney became more involved with infection the ureter became somewhat paler than it had been, but even on the 101st day it was obviously viable (see Figure 14).

On the 101st day the homograft site was explored. There was a well developed membrane about 3 mm. thick surrounding the kidney. Although it was loosely adherent to the kidney capsule, it could be stripped off the capsule with no great difficulty. The kidney was moderately en-

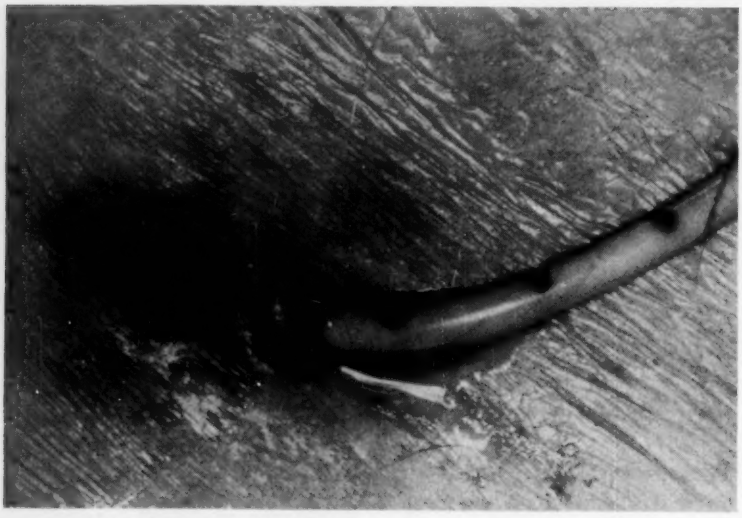


FIG. 14. B. B., CASE NO. 4—A CLOSE-UP VIEW OF THE HOMOGRAFT URETER ON THE 101ST DAY

The ureter is still viable, but appears paler than it did. Note that the mucosa has reached the skin.

larged, soft, and dark brown in color. It was obviously severely infected. The renal artery and vein and both anastomoses were still patent, and the capsule of the kidney bled when cut. The pedicle was clamped and the kidney was removed.

Grossly, the kidney showed multiple cortical abscesses and areas of infarction, with thromboses of some of the intrinsic vessels (see Figure 15). Microscopically, the pseudo-capsule surrounding the kidney was composed of a thick layer of dense fibrous connective tissue. The inner layer of this capsule, which lay against the renal transplant, was composed of a loose vascular stroma which resembled granulation tissue. There was extensive focal infarction of the kidney. In some areas thrombosis at the level of the arcuate or interlobular arteries, with subsequent infarction, had led to the formation of abscesses. In these areas the polymorphonuclear leukocyte was the predominant inflammatory cell. In other areas there was a prominent interstitial round cell infiltration. A few glomeruli showed changes resembling subacute glomerulonephritis, but this was not a striking lesion. The principle feature was that of focal infarction (see Figure 16).

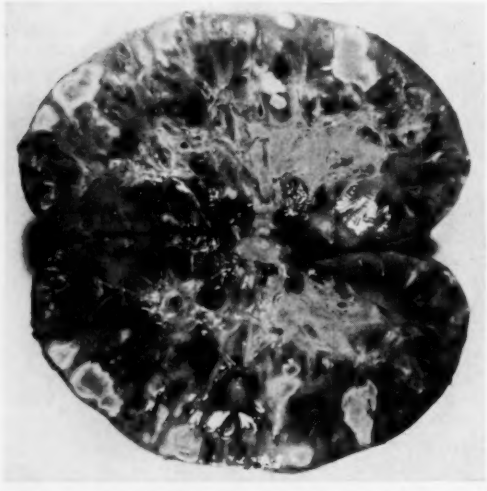


FIG. 15. B. B., CASE NO. 4—THE CUT SURFACE OF THE HOMOGRAFT, SHOWING AREAS OF INFARCTION AND CORTICAL ABSCESSES

The patient was discharged home on the 107th hospital day. There was some drainage from her leg wound for a month thereafter. She lived for 7 months, during which time a hemodialysis was performed on one occasion.

Case V—D. P., female, age 18, No. 9A816

Diagnosis: Chronic glomerulonephritis, hypertension.

The patient was an 18-year-old single girl whose fifth admission was necessitated by blurring of vision. A diagnosis of glomerulonephritis had been made 5 years ago. In spite of treatment, her symptoms had progressed.

Physical examination: On this admission the patient appeared pale and chronically ill. Blood pressure was 210/120. There were scattered ecchymoses over both

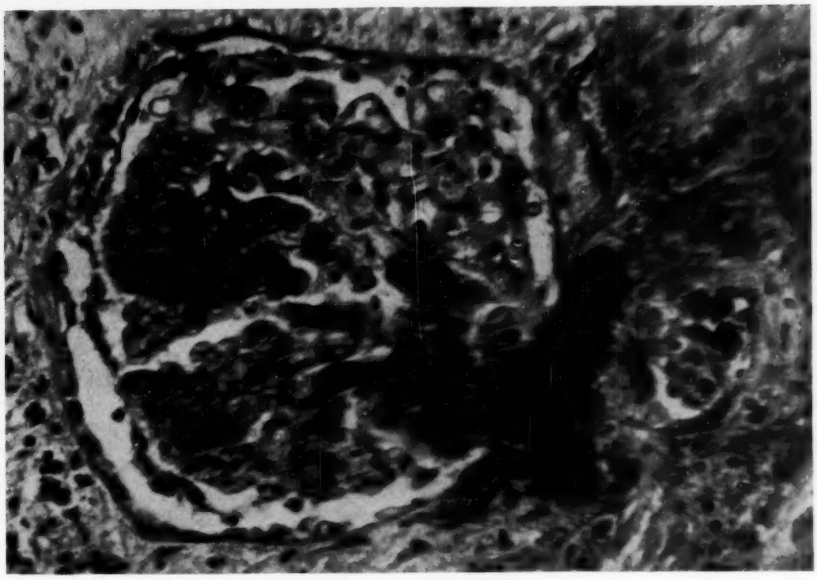


FIG. 16. B. B., CASE NO. 4—MICROSCOPIC APPEARANCE OF THE TRANSPLANT AT 101 DAYS, FOLLOWING ITS REMOVAL

This glomerulus is partially infarcted as the result of recent thrombosis of the afferent arteriole. The fibrin thrombus is densely basophilic and extends into capillaries at the hilum of the glomerular tuft. (Giemsa × 500)

lower extremities. The optic fundi showed fresh flame-shaped retinal hemorrhages, with old scars and exudate. The heart was enlarged to the anterior axillary line. There was a presystolic gallop rhythm. The lungs were clear. The liver edge was two finger-breadths below the costal margin. There was no ankle or sacral edema.

Laboratory data: Urine showed a 4 proteinuria. The spun sediment was loaded with red blood cells, and occasional white blood cells. Hematocrit was 21, blood urea nitrogen 97 mgm. per cent, total protein 4.9 gm. per cent, serum carbon dioxide combining power 17 mM. per L., chloride 112 mEq. per L., sodium 142 mEq. per L., potassium 6.5 mEq. per L.

Hospital course: Patient was placed on a rice diet and improved subjectively, although serum sodium values decreased to 126 mEq. per L. On 5-26-51 a skin flap was raised from the right thigh as a preparation for renal homotransplantation. An infection developed under this flap, however, and it could not be used.

On 6-6-51, a normal kidney was removed from a 2-year-old child during the course of an elective operation for relief of hydrocephalus.[7] The donor and recipient were of the same blood group (O, Rh +), and their bloods were compatible. The kidney was transplanted into the patient's left thigh, after a period of ischemia of 65 minutes. The arteries were anastomosed end-to-end, and the veins end-to-side. A double-ended pedicle flap was brought over from the lateral side of the leg, as in the previous case, and the defect was grafted with a split thickness graft from the other leg. The ureter was only 3 cm. long, and would not reach the skin, so a polyethylene tube was put in the ureter and brought out through a stab wound in the pedicle.

The transplant began to secrete urine at once, and during the first 24 hours following operation it secreted a total of 1,700 ml. of urine. The second 24 hours following operation only 153 ml. were elaborated. The urine was blood tinged. The urine urea nitrogen concentration was 177 mgm. per cent. Blood urea nitrogen at that time was 92 mgm. per cent. The patient tolerated the procedure well. Following this, there was only a small amount of bloody drainage from the homograft for a period of 12 days, during which time clots could be aspirated from the renal pelvis. The patient was given 200 mgm. testosterone propionate the first 24 hours, and was then maintained on 50 mgm. per day for 27 days.

Cortisone, 300 mgm. per day, was given for 8 days, and then gradually tapered off over the next 5 days. ACTH, 100 mgm. a day, was given from the 8th to 15th day and gradually tapered off over the next 4 days. Heparin was begun on the 2nd day with a dose of 100 mgm. and then continued until the 11th day at a level of 80 mgm. a day. Penicillin was given for the entire post-operative course at various dosage levels, and other

[7] Performed by Dr. Donald Matson.

TABLE VI

Case V—A comparison between the transplant and bladder urine on the 20th day *

	24-hr. Vol.	Cl	Cl 24 hr.	Creatinine	Creatinine 24 hr.	UUN	UUN 24 hr.
		mEq./L.	*mEq./L.*	*mgm. %*	*mgm.*	*mgm. %*	*grams*
Bladder	180	13	2.3	67	120	519	0.93
Transplant†	780+	33	25.8+	45	351+	615	4.8+

* BUN—128.
Cl—105.
† Urinary collections were incomplete due to leakage.

antibiotics were added from time to time, including Chloromycetin®, streptomycin, and Terramycin®.

After 12 days of almost complete anuria which followed the short-lived diureses, the kidney began to excrete urine again on the 14th day. The volume increased until 1400 ml. was obtained on the 23rd day. Maximum function occurred between the 14th and the 26th day. Thereafter the volume decreased until almost none was obtained on the 37th day. It was difficult to obtain accurate collections because of leakage around the catheter. Analysis of the urine from the transplant and that of the bladder urine on the 20th day is shown in Table VI. On that day the intravenous injection of 2 ml. of PSP resulted in prompt excretion of this dye from the homograft, but because of the difficulty in collection, this could not be quantitated.

Infection developed in a hematoma beneath the *right* leg flap. Cultures of paracolon *bacterium aerogenes* were obtained from this source, and from the bladder urine, transplant urine, and blood stream. The patient's general condition deteriorated rapidly from the 41st day on. She became comatose, developed EKG changes of hyperkalemia, and her blood urea nitrogen rose to 261 mgm. per cent. She died on the 49th day after transplantation.

Examination of the homograft at autopsy showed marked hemorrhage, infarction, and infection. There was frank abscess formation, and the inflammatory response was primarily that of polymorphonuclear leukocytosis. The pre-existing architecture of the kidney was almost entirely destroyed by these processes, but no evidence of glomerulonephritis could be found in the surviving glomeruli.

Case VI—B. L., male, age 26, No. 9B64

Diagnosis: Chronic pyelonephritis.

The patient was first admitted on 7-20-51 with a 7-year history of proteinuria. One year before admission a diagnosis of uremia with bilaterally contracted kidneys had been made. The blood NPN varied from 150 mgm. per cent to 230 mgm. per cent with a persistent anemia. He had several convulsions which had been controlled with Dilantin® before admission. Because of progressive uremia and intractable kidney disease, he was transferred from the West Roxbury Veterans Hospital to the Peter Bent Brigham Hospital for a renal homograft.

Physical examination revealed a small, pale, mentally dull young man in no distress. The optic fundi showed no changes. The lung fields were clear. The heart was not enlarged; there was a grade II basal systolic murmur. Blood pressure was 105/65. There were many spontaneous twitching movements of the extremities.

Laboratory data: Urine showed a 1+ protein. There were occasional red cells, white cells, and rare hyaline casts in the spun sediment. The hematocrit was 26. The white blood count was 3,350 with a normal differential. The blood urea nitrogen was 83 mgm. per cent, serum carbon dioxide combining power 11.3 mM. per L., chloride 106 mEq. per L., sodium 140 mEq. per L. and potassium 5 mEq. per L.

On 7-23-51 a previously normal functioning kidney removed from a 33-year-old woman at an elective operation for hydrocephalus [8] was transplanted into the patient's left thigh.[9] Donor and recipient blood types were both O, Rh +, and their blood was cross-matched and found compatible. The period of ischemia in this case was shortened to 14 minutes by performing the anastomoses over polyethylene tubes which were temporarily tied in place, after the technique of Hufnagel (56). Portions of the rectus femoris and sartorius muscles were removed to make room for the kidney, and no skin flap was used. The patient tolerated the procedure well, and 700 ml. of urine was elaborated from the homograft in the first 12 hours. This showed a urine urea nitrogen content of 800 mgm. per cent. Thereafter, however, no urine was produced by the kidney. The portion of the ureter projecting beyond the skin appeared necrotic within 24 hours. For 5 days there was no appreciable tension on the skin suture line, but after this time edema and tension became progressive.

Following the operation there was a marked rise in the blood urea nitrogen. The patient became confused and began to vomit. On the 3rd day, dialysis with the artificial kidney was accomplished with marked clinical and chemical improvement. The patient was treated with heparin 100 to 190 mgm. per day for 12 days, and 60 to 100 mgm. per day from the 14th day through the 18th day. No ACTH or cortisone were given. No testosterone was given until the 11th day at which time 50 mgm. per day was started and continued for 8 days. His course following operations was a septic one, with

[8] Performed by Dr. Donald Matson and Dr. Hathorne Brown.

[9] The transplantation was performed by Dr. Charles A. Macgregor.

a swinging fever rising as high as 103°, for a period of 51 days following operation. This febrile course was refractory to therapy with penicillin, Aureomycin®, Terramycin® and Chloromycetin®.

By the 14th day, the left leg had become tremendously swollen and tense. On that day, the patient had a generalized tonic, clonic convulsion similar to those prior to admission. Dilantin® therapy was again begun. There were no further convulsions, but the patient continued to vomit and remain drowsy. On the 14th day hemodialysis was again performed with good chemical and clinical results. The patient continued to run a septic fever. On the 18th day a retrograde injection of dye into the ureter of the homograft showed no definite calcyceal pattern and suggested "marked infection and necrosis." Blood culture on the 18th day was positive for enterococci and proteus vulgaris. On the 19th day, the homograft site was explored. The kidney itself appeared viable although it was surrounded by blood clots. Because of extreme edema of the skin and tension on the kidney an incision of the skin was made laterally and a flap of skin and subcutaneous tissue was swung medially to cover the transplant. The defect left by raising the skin flap was grafted by skin taken from the other thigh.

A *biopsy* of the kidney taken at operation showed marked ischemia, but no evidence of infarction or thrombosis. There was a marked interstitial round cell infiltration which was more prominent in this case than any of the others. The tubular changes again suggested those of an acute ischemic nephrosis ("lower nephron nephrosis"), with focal tubular degeneration and some regeneration. There were pigment casts present in several of the tubular lumena. The glomerular tufts showed no striking abnormalities. In some areas there was an accumulation of inflammatory cells around Bowman's capsule (see Figure 17). The kidney appeared viable.

Within a short period of time following operation, however, the wounds became foul and necrotic. Wound cultures grew enterococci and B subtilis, which were resistant to all but prohibitively large doses of the common antibiotics. *Proteus vulgaris, staph. aureous,* and *pseudomonas aerogenosa* were also grown from the wound. The swinging temperature continued. On the 49th day hemodialysis was again performed because of the deteriorating clinical condition, and on the 52nd day, since the homograft had produced no urine and appeared to be the obvious site for this infection, it was removed at operation. The homograft itself was covered with a thick, fibrinopurulent exudate, and there was complete distortion of the architecture with necrosis of the cortex and most of the medulla and pyramids. Thrombosis and infection were seen throughout the parenchyma.

Following the removal of the homograft there was a dramatic improvement in the signs of sepsis, and 5 days later the patient was transferred to the West Roxbury Veterans Hospital for further chronic care. Four hours after transfer, however, the patient suddenly exsangui-

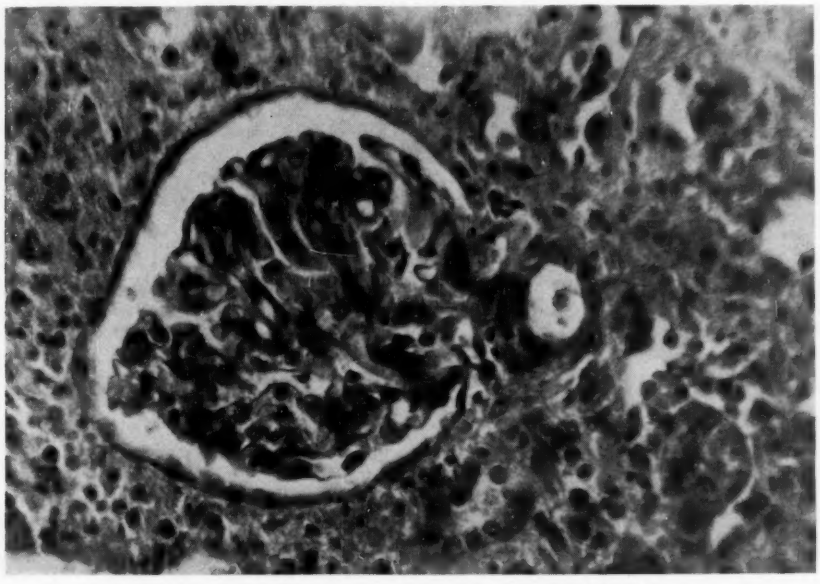


FIG. 17. B. L., CASE NO. 6—BIOPSY OF THE HOMOGRAFT ON THE 19TH DAY

The glomerular tuft shows no striking abnormalities. There is a conspicuous accumulation of inflammatory cells around Bowman's capsule. Tubules show focal degenerative changes. (H & E × 500)

nated from an eroded, infected portion of the common femoral artery just proximal to the bifurcation. Post-mortem examination[10] showed marked chronic pyelonephritis of the patient's own kidneys, and a rupture of the common femoral artery where the wall had been weakened by infection.

Case VII—D. G. female, age 28, No. 6E373

Diagnosis: Chronic pyelonephritis, hypertension.

The patient was first admitted on March 8, 1952. Two years before admission she noted the onset of decreased appetite and fatigue and had frequent upper respiratory infections. A year before admission she noted the onset of pallor, and six months before she had an acute episode of back pain. Two weeks before admission she was admitted to an outside hospital where a diagnosis of hypertension and kidney disease was made. Her treatment at this hospital included three transfusions of packed red cells. In spite of treatment, she began to vomit and had several convulsions. Because her condition continued to deteriorate, she was transferred to the Peter Bent Brigham Hospital for further treatment.

Physical examination at this time showed a thin, pale, weak woman who responded to commands, but was disoriented as to time and place. The skin was dry and yellow. There were several ecchymoses. There was a grade II precordial systolic murmur. Blood pressure was 190/70. The rhythm was regular. The abdomen was slightly distended but there was no peripheral edema. A chest film showed the heart to be of normal size.

Laboratory data: Urine showed 1+ protein and many white cells. Specific gravity 1.012. Culture of the urine showed four different coliform organisms. Urine urea nitrogen averaged 244 mgm. per cent. The 24-hour urine volume averaged 856 ml. The hematocrit on admission was 30 and the blood urea nitrogen, 284 mgm. per cent. The serum carbon dioxide combining power was 11.6 mM. per L., chloride 77 mEq. per L., sodium 124 mEq. per L., potassium 6.0 mEq. per L.

Dialysis on the artificial kidney was performed on two occasions with marked chemical and clinical improvement. She was discharged on the 21st hospital day. She was re-admitted on 4-16-52 to await kidney homotransplantation.

On May 7, 1952 kidney homotransplantation was carried out to the right thigh. The recipient was blood type O, Rh +. The donor was a 33-year-old woman with an intra-articular septal defect and hypertension who died during a cardiac operation. The donor's blood type was B, Rh +. Because of the incompatibility of the blood types, the kidney was perfused with 500 ml. of Ringer's solution to which 25 grams of albumin and 200,000 units of penicillin had been added.

There was an accessory artery leading to the lower pole of the donor kidney, and this had to be tied off. The left kidney of the donor was hypoplastic, and the right kidney, which was used for the transplantation, was slightly hypertrophied.

The arteries were anastomosed end-to-end, and the veins end-to-side. A double-ended pedicle flap was brought over as previously described, and the defect was split-grafted. When the clamps were taken off the blood vessels, the kidney became pink more slowly than the previous transplants, with the exception of the second one. This had also been performed with a kidney from a hypertensive donor of incompatible blood type.

After about a minute, it did become pink, though not quite as intensely pink as the previous ones. The area of the kidney supplied by the accessory artery remained dark blue. The blood supply of the ureter appeared adequate. It was brought out through a stab wound in the flap. A soft rubber catheter was placed in the ureter and led out to a bottle.

Post-operatively the patient was placed on heparin, 160 mgm. a day, for 48 hours. Penicillin was administered. No ACTH or cortisone was given. The patient received testosterone from the 12th to the 19th day. By the end of the first 48 hours a large hematoma had developed beneath the split-thickness skin graft. Attempts to evacuate the clot were only partially successful. Heparin was discontinued. There was no tension on the wound, because the wound had decompressed itself under the skin graft.

By evening of the 3rd day the patient had developed severe potassium intoxication. This was treated by peritoneal lavage because of fear of increasing hemorrhage and potassium absorption if heparinization and hemodialysis were used (76). The patient made a good temporary response to this, but by the fifth day she again showed severe hyperkalemia. A dialysis on the artificial kidney was performed at this time. Later this same day the patient was taken to the operating room and, under a small amount of nitrous oxide, the hematoma under the graft was evacuated and the wound packed.

Peritoneal lavage was necessary again on the 13th day, for hyperkalemia.

The homograft excreted a few ml. of blood-tinged urine at first, but by the end of the first 24 hours the urine was clear. By the end of the 3rd day a total of 100 ml. had been collected. The ureter appeared viable at this time. On the 4th day an infarct of the ureter had apparently developed and the urine was again slightly blood tinged. On the 5th and 6th days the urine became more bloody, and by the 7th day urine output had ceased. At no time was the 24-hour volume more than 50 to 60 ml., but the course of this transplant differed from that of the "anuric" phase of compatible blood type grafts in that the urine became bloody again after it once cleared, and then ceased flowing entirely, instead of gradually increasing in amount and staying clear.

On the 17th day the homograft was removed. Both vessels were occluded by thrombus with infarction of the kidney. There was no infection, and no appreciable blood clot around the kidney. The host's vessels were patent right up to the anastomoses. Histologically there

[10] We are indebted to the Pathology Department of the West Roxbury Veterans Hospital for the results of this examination.

were changes in the walls of the large blood vessels of the transplant, and generalized infarction of the kidney with extensive calcification. There was no evidence of intrinsic inflammatory or proliferative change (see Figure 18).

Despite peritoneal dialysis on the day of operation, the patient died 2 days later. At post-mortem examination severe pyelonephritis with almost total destruction of the patient's kidneys was found.

Case VIII—O. F., female, age 57, No. 7E213

Diagnosis: Periarteritis nodosa with complete anuria.

This 57-year-old woman was admitted to the Peter Bent Brigham Hospital for the first time on 8-22-52 with a chief complaint of anuria of 13 days' duration. She was well until four years before when she first noted onset of asthma, which increased in severity so that two years before she had to be hospitalized for oxygen therapy. In November, 1950 she received 4 days of ACTH therapy with excellent results. In November, 1951, 9 months prior to admission, she received a 2-weeks' course of "Pyromine." Following the last injection of this substance she noted the rapid onset of pain, weakness, numbness, coldness of the extremities, and periorbital edema, associated with a temperature of 101. She was admitted to an outside hospital where a consultant felt that asymmetrical peripheral neuropathy suggested the diagnosis of periarteritis nodosa. A biopsy of the right gastrocnemius muscle confirmed this impression. A blood urea nitrogen at this time was 6 mgm. per cent. She was given ACTH 30 to 90 mgm. a day from December, 1951 to July, 1952, with relief of her peripheral neuritis and asthma.

Three weeks prior to entry, following excessive exposure to the sun, the patient developed a temperature of 100.8 and became fatigued and nauseated. Her urinary output gradually diminished until she became essentially anuric 13 days prior to entry. Ten days prior to entry she was admitted to an outside hospital. Retrograde pyelograms were negative. Her total urinary output for 10 days was 20 ml. Her blood urea nitrogen rose from 126 to 175, and she was transferred to the Peter Bent Brigham Hospital for dialysis.

Physical examination: On admission the patient was a pale, middle-aged woman lying quietly in bed without complaint. Blood pressure was 155/75. The optic fundi were unremarkable. The heart and lungs were normal. The abdomen was moderately distended, and fluid wave was present. The liver was felt to be somewhat enlarged. There was 2+ pitting edema of the extremities and sacrum. The deep tendon reflexes were hyperactive. There was a positive Babinski on the left. Vibration sense was diminished over the left lower leg, and there was hyperalgesia of the left foot.

Laboratory data: Blood urea nitrogen 264 mgm. per cent, carbon dioxide combining power 14.6 mM. per L., chloride 99 mEq. per L., sodium 136 mEq. per L., potas-

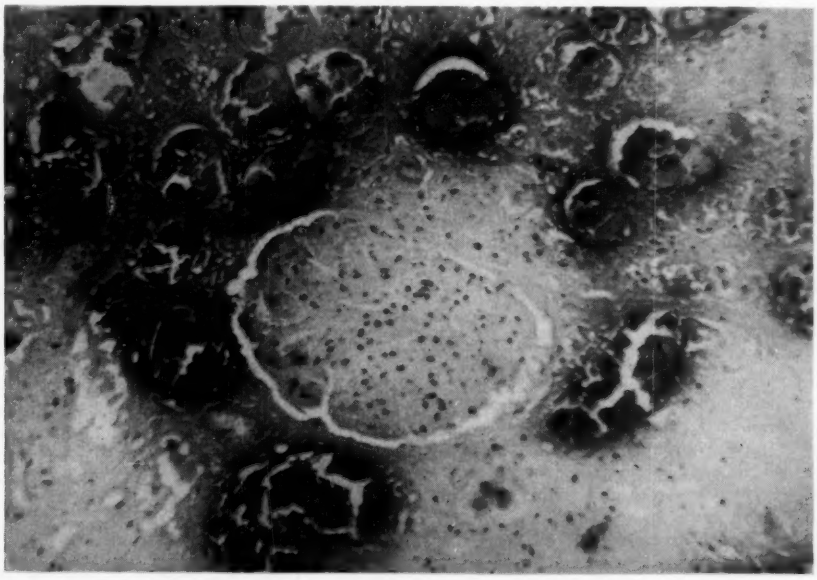


FIG. 18. D. G., CASE NO. 7—MICROSCOPIC APPEARANCE OF THE TRANSPLANT AT POST-MORTEM

The outlines of an infarcted glomerulus can be identified. There is no evidence of intrinsic inflammatory or proliferative change. Adjacent tubules show striking degeneration and calcification of epithelium. (H & E × 250)

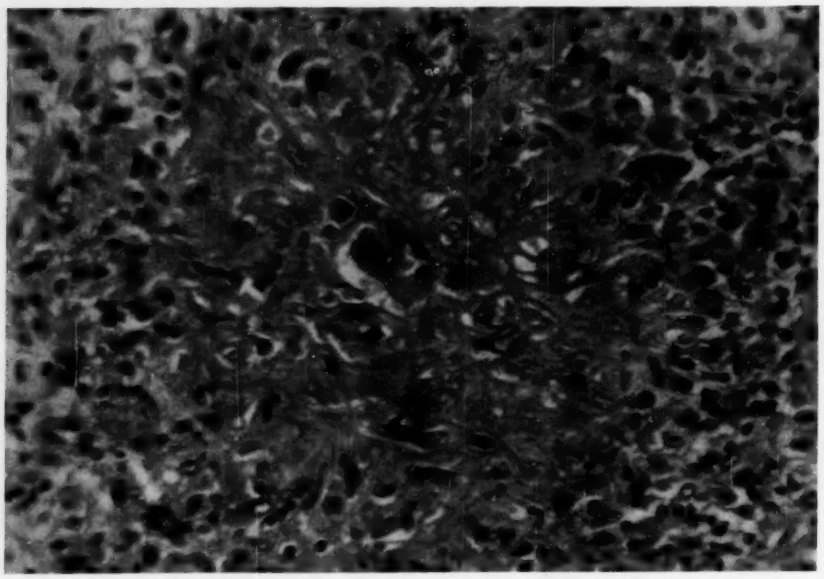


FIG. 19. O. F., CASE NO. 8—A BIOPSY OF THE LEFT KIDNEY OF THE PATIENT TAKEN ON THE 41ST DAY OF ANURIA, 4 DAYS BEFORE RENAL HOMOTRANSPLANTATION WAS DONE

The glomerular architecture is virtually obliterated by the proliferative and inflammatory changes. At the periphery inflammatory cells are present in moderate numbers. (H & E × 500)

sium 6.1 mEq. per L., hematocrit 19, white blood count 8,600 with 89 per cent polymorphonuclear leukocytes.

Hospital course: A catheter was inserted in the femoral vein and the patient was begun on 50 per cent glucose 400 to 700 ml. a day. On the 3rd day she had a convulsion, and on the following day hemodialysis was carried out with good clinical improvement. She continued to be anuric, and she was again dialyzed because of progressive signs of uremia on 9-4-52 and on 9-16-52, each time with good response. On 9-18-52 a biopsy of the left kidney was carried out.[11] This showed severe renal damage. The proliferative and inflammatory changes were so pronounced in some areas as virtually to eliminate the glomerular architecture (see Figure 19). A moderate number of inflammatory cells were present around the glomeruli. The picture was felt to be consistent with marked periarteritis of the kidney. On 9-22, the 45th day of anuria, a kidney homotransplant to the left thigh was performed. The donor was a patient with ruptured sinus of Valsalva who died during a cardiac operation. The blood types of the donor and recipient were both O, Rh +, and their bloods were compatible. The left kidney of the donor was used, and an end-to-end anastomosis was made between the profunda femoris artery and the renal artery, and an end-to-side anastomosis was made between the renal vein and the common femoral vein. The kidney was not perfused. The period of ischemia was 275 minutes.[12] No heparin was injected. Circulation through the kidney appeared excellent, and the color of the transplant became normal as quickly as any we have done. Peristaltic waves were seen in the ureter, and the blood supply to the tip of the ureter appeared normal.

A pocket was created medially in the usual fashion, and a double-ended pedicle flap was used to cover the kidney. The fascia lata was covered with a split thickness graft taken from the abdomen. The ureter was brought out through a stab wound in the flap, and a soft rubber catheter was inserted in it.

Post-operatively the patient was treated with heparin 90 mgm. in the first 18 hours, then 160 mgm. per day for 7 days, gradually tapering it off from the 7th to the 10th day. She was placed on ACTH 100 mgm. a day I-M for 5 days, then 25 mgm. I-V over an 8-hour period for 2 days, and then 20 mgm. ACTH gel twice a day through the 19th day. It was gradually tapered off from the 19th to the 22nd day. The first 24 hours she was given 200 mgm. testosterone, and thereafter she received 50 mgm. a day for 12 days, and 50 mgm. every other day

[11] This operation was performed by Dr. J. Hartwell Harrison.

[12] This long period of ischemia was occasioned by a delay in obtaining the donor kidney.

FIG. 20. O. F., CASE NO. 8—APPEARANCE OF THE CUT SURFACE OF THE RENAL TRANSPLANT AT POST-MORTEM
Note the stippled appearance of the cortex.

from the 13th to the 38th day. Penicillin, streptomycin, and Terramycin® were given at various times.

The patient's only food, except for an occasional cup of tea, was 200 to 700 ml. (usually 400 to 500 ml.) per day of 50 per cent glucose given I-V *via* the femoral catheter over the course of 24 hours. She received one transfusion of washed red blood cells on the 10th day. For 83 days she had essentially no protein or fat intake. Daily electrocardiograms were taken, and these plus the patient's clinical status were used to determine when hemodialysis was to be done.

There was no edema of the leg in which the kidney was implanted, although a hematoma formed under the split-thickness skin graft. The wound through which her own kidney had been biopsied never entirely healed, nor did the skin graft donor site or the skin graft heal. The wound in the thigh only partially healed, there being some necrosis of the upper aspect. A small collection of pus was aspirated through a needle from the lower end of the leg wound, but there was surprisingly little infection present.

By the 3rd post-operative day the end of the ureter had become black and dry. Only a few milliliters of bloody fluid came through the catheter, and no function of the transplant developed.

The patient ran a sub-normal temperature (95.2° to 97.2°) from the time of the operation until the 6th post-operative day, when she was dialyzed. Her temperature rose to 98.6° following the dialysis, and persisted at a

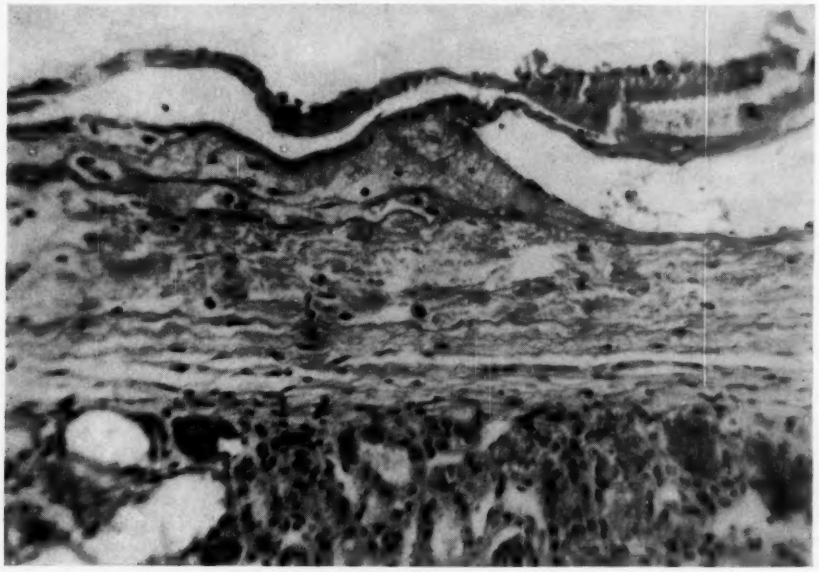


FIG. 21 O. F., CASE NO. 8—MICROSCOPIC APPEARANCE OF THE CAPSULE OF THE TRANSPLANTED KIDNEY AT POST-MORTEM
Around the capsule there is a thin layer of fibrin, with only occasional inflammatory cells, and no proliferating fibroblasts.

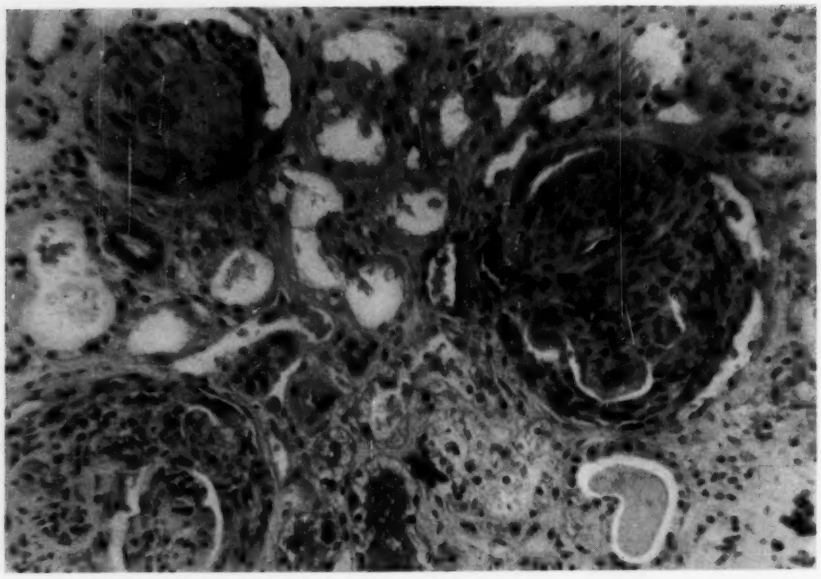


FIG. 22. O. F., CASE NO. 8—MICROSCOPIC APPEARANCE OF THE TRANSPLANT AT POST-MORTEM

The glomeruli show striking proliferative changes with resulting "crescent" formation, and obliteration of capillaries. Adjacent tubules exhibit vacuolated epithelium, and some contain recently extravasated blood. (H & E × 250)

low normal range (96.6° to 98.6°) until the 2nd post-operative dialysis on the 15th day, at which time it rose to 99.8°. It then remained within normal range until the 32nd day, at which time it again became sub-normal. It gradually went down, until during the last 24 hours it was too low to record on standard clinical thermometers. The patient died quietly on the 38th post-operative day, the 83rd day of complete anuria, the 23rd day since the last dialysis. Prior to the 22nd post-operative day the patient was communicative and pleasant, reading during the day, and having no complaints. On the 22nd day she began having seizures, followed by semi-coma. The blood urea nitrogen at that time was 119, serum potassium 5.1, hematocrit 19. From this time until the time of death the patient was semi-comatose. A blood urea nitrogen on the 32nd day was 204, and a white blood count and hematocrit on this day were 3,850 and 15, respectively. An electrocardiogram on the 33rd day showed no evidence of hyperkalemia, but one taken on the 38th day shortly before death showed definite changes of hyperkalemia.

At post-mortem examination there were several very remarkable findings. The kidney biopsy site and the skin graft donor site on the abdomen were very poorly healed. The skin graft over the lateral aspect of the leg had completely sloughed away. The medial leg wound had only weakly healed, and there was some necrosis at the upper end.

When the wound was opened it was very easy to strip the subcutaneous tissues away from the kidney, there being very little reaction between host and the graft. The blood vessels were easily identified, and there was very little reaction around them. There was a small, completely organized, non-occluding clot in the common femoral vein just below the inguinal ligament where a bulldog clamp had been applied during the operation. The transplant appeared normal in size, and there was no significant infection around it.

The cut surface of the kidney appeared normal, except that there were multiple pin-point, brownish-red spots scattered throughout the cortex (see Figure 20). The patient's own kidneys showed almost no recognizable normal architecture on cut section.

The microscopic findings were as follows:

A. The transplanted kidney: Unlike Cases No. 1 and No. 2, there was no pseudocapsule present (see Figure 21). There were no thromboses or infarcts present. There was a striking proliferation of the parietal epithelial layer of Bowman's capsule and of the epithelium of adjacent capillary loops, leading to marked "crescent" formation. The rest of the tuft was contracted and relatively bloodless (see Figures 22 and 23). These findings

were compatible with a severe acute glomerulonephritis. The tubules showed vacuolation of the epithelium, and in some instances there was recently extravasated blood in the lumen. There were areas of tubular degeneration with calcification and also of tubular repair. There was very little round cell infiltration.

B. The contralateral donor kidney: At the time of transplantation both of the donor's kidneys appeared similar, and grossly normal. The kidney which was not used for transplantation was examined microscopically. It appeared normal, and there was no evidence of glomerular disease.

C. The patient's own kidneys: Proliferative and inflammatory changes had almost completely destroyed the glomeruli. The tubules were atrophic. Periglomerular inflammatory cells were less conspicuous than in the biopsy specimen (see Figure 24).

Comment: The transplanted kidney had developed, in 38 days, glomerular changes of such magnitude that it was never able to elaborate urine. Presumably these glomerular changes began during the anuric phase following transplantation, and were well enough developed at the end of this time to prevent recovery of function. No changes diagnostic of periarteritis were seen in the transplant, but the large doses of ACTH post-operatively may have been enough to arrest these, as it apparently had elsewhere in the patient. It failed to halt the glomerular damage, however, just as it had failed in the case of the patient's own kidneys.

The failure of this recipient to react violently locally to the transplant is in marked contrast to Case No. 4. This may have been due to the protracted anuria and lack of adequate caloric and protein intake, and it correlates well with the failure of the wounds to heal, and the lack of clotting in the blood vessels. On the other hand, the severe glomerular changes are in marked contrast to the other cases reported here, where tubular destruction, thromboses of small blood vessels within the kidney substance, and infection were the predominant changes. Since this patient had a severe, generalized, allergic disease, it is tempting to postulate that these glomerular changes were the consequence of this disease.

Case IX—G. W., male, age 26, No. 7E503

Diagnosis: Chronic glomerulonephritis and hypertension.

This 26-year-old male physician first entered the Peter Bent Brigham Hospital on October 17, 1952 with the complaint of chronic glomerulonephritis. His illness began at the age of 12 with an upper respiratory infection, which was followed in a few days by microscopic hematuria and albuminuria. One and one-half years prior to

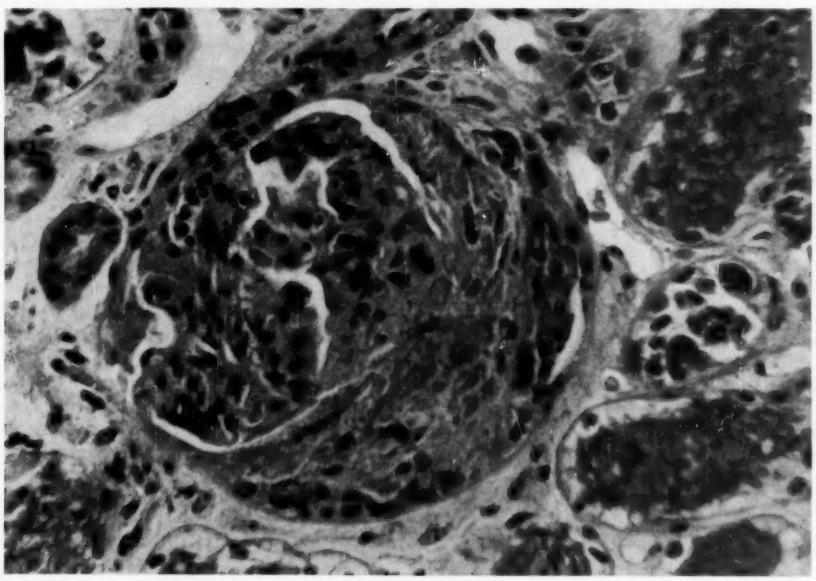


FIG. 23. O. F., CASE NO. 8—MICROSCOPIC APPEARANCE OF THE TRANSPLANTED KIDNEY AT POST-MORTEM

There has been a striking proliferation of the parietal layer of epithelium of Bowman's capsule, and of the epithelium of adjacent capillary loops, with resulting "crescent" formation. Elsewhere the tuft is contracted and relatively bloodless. The epithelium of nearby tubules is vacuolated, and recently extravasated blood fills the lumens. (H & E × 500)

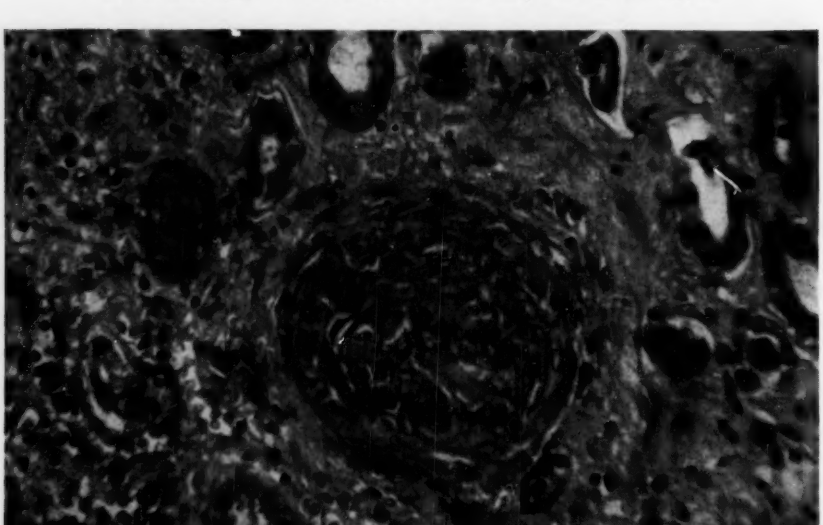


FIG. 24. O. F., CASE NO. 8—THE MICROSCOPIC APPEARANCE OF THE PATIENT'S OWN KIDNEY AT POST-MORTEM

The glomerulus in the center of the picture is obliterated and adjacent tubules are atrophic. Inflammatory cells in the periglomerular region are less conspicuous than in biopsy specimen. (H & E × 500)

admission he was found to have a blood pressure of 160/100 and a blood urea nitrogen of 30. At this time he noted the onset of ankle edema at night and moderate periorbital edema. Two months prior to admission he had a severe headache and developed visual changes which were found on funduscopic examination to be due to retinal hemorrhages. He was hospitalized elsewhere at this time and his blood urea nitrogen was found to be 114 mgm. per cent, and his blood pressure 200/120. He had several days of gross hematuria and noted occasional nausea and vomiting. He was given three transfusions and was placed on a low protein, low salt diet. He entered the Peter Bent Brigham Hospital for a consideration of renal transplantation.

Physical examination: The patient was a pale young man with definite periorbital edema. Blood pressure was 188/118. The optic fundi showed A-V nicking with arteriolar narrowing and irregularity. Both discs were blurred, and there were hemorrhages and exudates in both eyes. Lungs were clear. There was a Grade II apical systolic murmur. There was generalized lymphadenopathy.

Laboratory data: Blood urea nitrogen 65 to 80 mgm. per cent, carbon dioxide combining power 23.4 mM. per L., chloride 99 mEq. per L., sodium 130 mEq. per L., potassium 5.3 mEq. per L., hematocrit 32, white blood count 9,200 with a normal differential. Urinalysis revealed a specific gravity of 1.006, and there was 2+ proteinuria. Microscopic examination revealed 40 to 60 red blood cells and 8 to 10 white blood cells per high power field. There was fine granular casts. PSP excretion was 7 per cent in two hours. A chest x-ray was negative.

Hospital course: On November 6, 1952, the 21st hospital day, an operation was carried out to outline the skin flap on the right leg in preparation for doing a renal homotransplant. It was decided to create the flap in advance on this patient because his thigh was very thin and it was felt that the pedicle graft would not stand up under the increased tension that is developed when a renal transplant is placed in the thigh. The patient was discharged eight days later. Sixteen days later the patient was re-admitted and on December 3, 1952 the skin flap was elevated and then laid back down and resutured in place. The blood urea nitrogen prior to this operation was 76. Four days after this operation the patient was discharged again to await kidney transplantation.

He was re-admitted on February 11, 1953 and a renal transplantation to the right thigh was carried out at this time. The donor was a patient with mitral and aortic stenosis who died during a cardiac operation. The blood types of both the donor and recipient were A, Rh+, and their bloods were compatible. Although we prefer to put the right kidney in the right thigh, the blood vessels of this kidney were not suitable for use and therefore

the left kidney was used. The kidney was placed in a polyethylene bag, which had been made especially for this purpose, and the artery, vein, and ureter were each brought out through small holes in the bottom of the bag. An end-to-end anastomosis was done between the profunda femoris and the renal artery, and an end-to-side anastomosis was made between the renal vein and the common femoral vein. The kidney was not perfused. The period of ischemia was 180 minutes. No heparin was injected. Circulation through the kidney appeared excellent. Peristaltic waves could be seen in the ureter, and the blood supply to the tip of the ureter appeared excellent. After all the bleeding points which could be seen on the kidney capsule were secured, the open end of the bag was sealed shut with the cautery. The bag was loose around the kidney and in no way constricted it (see Figure 25).

A pocket was created medially in the usual fashion and the previously outlined skin flap was again raised and was used to cover the kidney. The fascia lata was covered with two split-thickness grafts taken from the left thigh. The ureter was brought out through a stab wound

FIG. 25. G. W., CASE NO. 9—THE DAY OF OPERATION

The homografted kidney has been sealed in a polyethylene bag. On the top (A) the anastomosis can be seen between the renal artery and the profunda femoris artery. The femoral artery is being retracted. The ureter may be seen lying on the gauze sponge at the right-hand side of the picture. On the bottom (B) is a view of the end-to-side venous anastomosis.

in the flap and a soft rubber catheter was inserted in it. Because the left kidney was used, a U-shaped loop had to be made in the ureter. There was a large amount of oozing from the wound and therefore a perforated catheter was brought out through the lower end of the medial incision and connected to Wangensteen suction.

Post-operatively the patient was given 200 mgm. of testosterone in the first 24 hours and then 50 mgm. daily from then on for the next 62 days. No ACTH, cortisone, or heparin were given. Penicillin, streptomycin, Aureomycin®, and Terramycin® were used at various times. The patient was placed on Terramycin® on the 29th post-operative day and was maintained on a dose of 1.0 gm. a day to the 145th day.

For the first two days post-operatively there was profuse bloody drainage from the operative site through the catheter that had been placed in the wound. By the third day the drainage had almost stopped, and the catheter was removed to prevent infection. The bleeding had not stopped, however, and although the patient was ordinarily hypertensive, by the 5th day his blood pressure had fallen to shock levels. His hematocrit, which had been 32 on the 2nd day, had fallen to 17 by the 5th day. His thigh was moderately swollen. The patient was given protamine sulfate (although no heparin had been used), vitamin K, and fresh blood transfusions in an effort to decrease his tendency to bleed. For the next two days he continued to ooze, however, and by the morning of the 8th day he was in a precarious state. He had received seven blood transfusions. His hematocrit, which had been raised to 22 with the transfusions, had again dropped to 17. His transplant had produced no urine, and the output of his own kidneys had fallen to 300 ml. (total) for the preceding two days. His thigh was tremendously swollen. The blood urea nitrogen had risen to 166 mgm. per cent, and the serum sodium had fallen to 124 mEq. per L.

The dressings were entirely removed from the leg. The suture line between the split-thickness skin grafts and the pedicle flap had been separated and a large blood clot was lifting the pedicle flap off the anterior thigh muscles and the renal transplant. The tip of the ureter still appeared to have excellent circulation, however, and the two split skin grafts had entirely taken, having been saved by a stint which was tied in place over them. The pedicle flap, also, was of excellent color. The lateral portion of the hematoma was lifted out of the opening in the wound, and its volume was estimated at about 1000 ml. After its removal the transplanted kidney in its polyethylene covering could be seen presenting in the opening in the lateral aspect of the wound, pushed over by hematoma medial to it.

The medial wound was re-opened at operation. Blood clot amounting to an estimated 2000 ml. was removed. The kidney and ureter appeared viable. A small rip, about 2 cm. in length, had occurred in the margin of the polyethylene bag which had been sealed with the cautery. An adhesion between subcutaneous tissue and kidney capsule had occurred through this opening. The adhesion was broken. The transplant was placed back in its medial position. The lateral margin of the pedicle flap was resutured to the split graft. The wound oozed from every surface, and it was impossible to control the bleeding completely. The medial portion of the wound was closed around three Penrose drains. The catheter was left in the ureter.

Following this operation, the patient's condition remained poor for several days. His blood urea nitrogen rose to 244 mgm. per cent on the 19th day after transplantation, the 11th day after the evacuation of the hematoma. Gradually the bleeding from the wound slowed down and stopped, and the drains were removed 15 days after the second operation. At this time the polyethylene bag could be seen through one of the drain holes.

For 11 days the transplant put out no urine. Then from the 12th to the 18th day it put out from 5 to 26 ml. daily. On the 19th day it began to "open up," producing 75 ml., and for the next 5 days it produced gradually increasing amounts. From the 24th to the 30th day it reached a plateau of around 400 ml. daily. This was interesting in view of the fact that the other three transplants with measurable function reached their maximal urine output from the 13th to the 28th day. The output continued to increase until, on the 37th day, a 24-hour output of 1,000 ml. was reached, and from this time on the transplant elaborated 1,000 to 3,000 ml. a day until the sudden events which led to the patient's demise 6 months after the transplantation.

On the 28th day the catheter was removed from the ureter, since peristaltic waves were producing leakage of urine around the catheter. A ureterostomy cup was glued to the skin around the ureter, and the urine drained from here into a bag which was strapped to the patient's thigh (see Figures 26 and 27). The cup was changed every three weeks.

There was a considerable amount of thin purulent drainage from the drain sites in the medial wound. This cultured micrococci and *A. aerogenes* which were somewhat sensitive to Aureomycin® and Terramycin®. The patient was given both drugs at various times, but proved to tolerate Terramycin® better, and so was maintained on this until the 145th day. The three drain sites healed until only a small opening was present at the superior site, just below the inguinal ligament. A small amount of drainage persisted at this location until the 148th day, at which time all drainage stopped and the wound closed entirely.

The patient was gradually mobilized. His appetite returned, and he gained weight. His blood urea nitrogen decreased and his hematocrit rose. He learned to take care of his ureterostomy very well. On the 81st day after the transplantation he was discharged home. From this time on, he returned every two to three weeks for brief periods of hospitalization for study. His appetite remained good, and he ultimately felt better than he had for years.

Kidney function studies were done at frequent intervals. They are summarized in the following paragraphs, together with notes of other observations which were of interest to us.

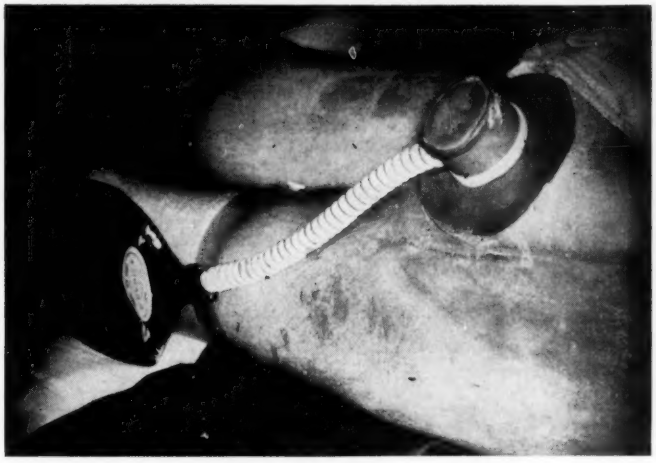


FIG. 26. G. W., CASE NO. 9—LATERAL VIEW OF THE RIGHT THIGH

The ureterostomy cup is glued in place, and the collecting bag is strapped to the patient's leg. The graft site can be seen just beneath the cup.

1. Excretion of metabolites and electrolytes

In Table VII a comparison is made between transplant and bladder urine at representative periods throughout the patient's course, together with blood studies during the same period. It may be seen that the transplanted kidney, once its function reached a steady plateau, excreted more metabolites than both host kidneys. On the 65th day, for example, the patient's own kidneys together put out 2.4 gm. of urea nitrogen in 24 hours, while the transplant excreted 6.8 gm. The total urea nitrogen output was 9.2 gm., which corresponds to an output of about 18.4 gm. of urea. This would be at the lower limits of normal range (15 to 35 gm.) on an average protein intake. The urea clearance of the transplant (x2) on this day was 16 ml. per min. The function of the transplanted kidney with respect to the clearance of urea was thus about 25 per cent of normal.

On the 74th day the total 24-hour creatinine excretion in the bladder urine was 253 mgm., while in the urine from the transplant it was 1,000 mgm. The normal 24-hour output of creatinine for a man of this size is 1.2 to 1.7 gm.

The total nitrogen output of the transplanted kidney consistently ran about twice that of the total output in the bladder urine.

The potassium excretion of the transplanted kidney was greater than that in the bladder urine, and was within normal limits. The sodium and chloride excretion of both the transplant and bladder urine was quite low.

The pH of the bladder urine ran between 4.7 and 5.3. The pH of the transplant urine began at 5.6 on the 37th day and rose to 7.2 by the 71st day.

There was no sugar in the urine.

2. Concentration and dilution tests

The specific gravity of the bladder urine was about 1.007, and that of the transplant varied between 1.005 and 1.012. A concentration test carried out on the 53rd day showed no significant ability of either the transplant or the host kidney to respond with increases in specific gravity. The specific gravity of host and transplant urine fell with increased fluid intake. (See also Pituitrin® test).

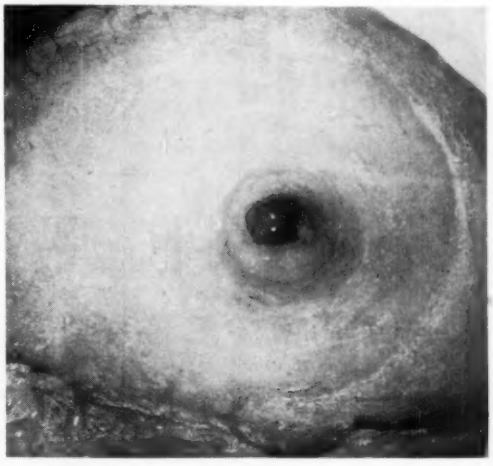


FIG. 27. G. W., CASE NO. 9—VIEW OF THE URETEROSTOMY WITH THE CUP REMOVED

TABLE VII

G. W. Case No. 9—A comparison between transplant and bladder urine at representative periods throughout the patient's course, together with blood and serum studies over the same period

	Urine														Blood and serum						
	Volume		Urea nitrogen *gm./24 hr.*		Creatinine *mgm./24 hr.*		Uric acid *mgm./24 hr.*		Chloride *mEq./L.*		Sodium *mEq./L.*		Potassium *mEq./L.*		BUN *mgm. %*	Uric acid *mgm. %*	CO_2 *mM/L.*	Cl *mEq./L.*	Na *mEq./L.*	K *mEq./L.*	Hematocrit
Day	B*	T*	B	T	B	T	B	T	B	T	B	T	B	T							
2	510	0													89		20.0	102	132	5.0	32
5	650	0													138		21.5	79	125	5.0	17
8	1,350	0													166		21.2	87	124	4.9	17
15	2,075	5													218		21.6	81	121	4.8	27
19	1,750	75													244		24.3	87	128	3.6	27
25	1,600	380	8.8	2.2							13	9	25	13							
26	1,700	385													214		24.8	92	128	3.4	22
33	1,500	520													173		23.0	101	135	4.3	31
34	1,300	580	7.1	3.6	949	580					22	15	31	32							
40	1,720	1,200			860	804									129		20.9	103	137	4.5	33
43	1,000	1,080	4.4	6.8	644	848			47	26	30	34	25	26							
48	950	1,200	4.3	6.6	687	882	373	690	46	28	24	25	35	43	81		17.5	106	138	5.4	38
54	600	1,050	2.9	9.0			247	718	17	20	11	19	18	34	70						33
59	713	1,286			512	860	195	800	29	32	17	18	20	37	80	8.3	18.3	101	140	3.6	
65	825	1,400	2.4	6.8	462	764			32	29	18	20	20	41	62	7.0	19.9	110	130	4.0	
72															71	7.0	22.1	103	133	4.1	33
74	550	2,200			253	1,000															
90															60	7.6	24.0				30
103															73	7.3	17.9	107	135	4.0	31
123					280	930	280	1,200							85		16.7	109	138	3.8	43
153															34†		20.0		135		
155															43	6.8	16.0	107	136	3.4	41

* B: Bladder urine.
T: Transplant urine.

† The blood studies on this day were done in the renal laboratory. All other blood determinations were done in the routine laboratory.

TABLE VIII

G. W. Case No. 9—4-10-53—Renal functional studies carried out on the 58th day *

Serum		Urine			
		Bladder		Transplant	
		Mean	S.D.	Mean	S.D.
pH	7.32	5.5	(±.26)	6.9	(±.41)
Hct.	33	32.5	(±2.65)	31.5	(±3.32)
BUN (mg. %)	60	4.9	(±.25)	6.3	(±.3)
CO_2 (mM/L.)	20	17.2	(±.32)	22	(±.32)
Na (mEq./L.)	136	34		19	
		17		42	
		19		30	
K (mEq./L.)	3.35	25		30	
PO_4 (mM/L.)	1.3	6.9		8.5	
		.96		.96	

C inulin (ml./min./1.73 M^2)	
C PAH (ml./min./1.73 M^2)	
C urea (ml./min.)	
Filtration fraction	
Na conc. (mEq./L.)	
Cl conc. (mEq./L.)	
NH_3 (mg. %)	
K conc. (mEq./L.)	
PO_4 (mM/L.)	
Average volume (ml./min.)	

* The clearance of inulin, PAH, and urea are the mean value for four 20-minute periods shown with the standard deviation. Serum values are those at the beginning of the clearance periods.

TABLE IX

G. W. Case No. 9—5-27-53—Renal function studies carried out on the 103rd day

Serum		Urine			
		Bladder		Transplant	
pH	7.36	4.1	C inulin (ml./min./1.73 M^2)	7.9	
PO_4 (mM/L.)	1.4	23	C PAH (ml./min./1.73 M^2)	31	
BUN (mg. %)	48	3.9	C urea (ml./min.)	7.9	
CO_2 (mM/L.)	21.4	18	Filtration fraction	25	
Cl (mEq./L.)	109	1.06	Average volume (ml./min.)	1.45	
Na (mEq./L.)	140	41	Na conc. (mEq./L.)	19	
K (mEq./L.)	4.2	14	K conc. (mEq./L.)	22	

3. PSP and aminophyllin tests

Phenolsulphonephthalein tests were done on two occasions. On the 28th day the total output of dye in two hours' time from the bladder was 2 per cent, and from the transplanted kidney 4 per cent. On the 39th day the bladder urine contained 7.5 per cent, and the transplant 5 per cent. The urinary output of the transplanted kidney increased markedly following the I-V injection of aminophyllin.

4. Clearance studies[13]

Studies of renal clearance were done on three separate occasions, (Tables VIII, IX, and X), on the 58th, the 103rd, and the 153rd day, respectively. While the clearance of inulin and PAH are difficult to interpret in renal disease as far advanced as this, nevertheless under comparable circumstances, the function of the transplanted kidney in these parameters was better than both of the host kidneys combined. It is also of interest that the function of the transplant was continuing to improve at least as late as the 153rd day, when the last test was done. The lowering of the blood urea nitrogen further attests to the fact that the transplanted kidney was improving the patient's excretory ability.

5. Pitressin® test [14]

On the 50th day a Pitressin® test was carried out in the following manner: With a water load of 20 ml. per Kg., maximal diuresis ensued. This amounted to 2.0 ml. per min. for the patient's own kidneys (combined), and 3.0 ml. per min. for the transplant. The specific gravity of both urines fell from 1.007 to 1.001. After the control period, 15 milliunits of Pitressin® was given intravenously. The maximal decrease in minute output amounted to 65 per cent for the patient's own kidneys, and 70 per cent for the transplanted kidney. The maximal specific gravity was 1.006 for the patient's own kidneys and 1.010 for the transplant. The chloride concentration of the bladder urine increased from an average of 26.5 mEq. per L. during maximal diuresis to a maximum of 37.0 mEq. per L. following the administration of Pitressin®, and that of the transplant increased from an average of 16.9 mEq. per L. to a maximum of 33.4 mEq. per L.

[13] These were kindly carried out for us by Dr. John Finkenstaedt.

[14] We are indebted to Dr. Joseph Dingman for this study.

TABLE X

G. W. Case No. 9—7-14-53—Renal function studies for the transplanted kidney on the 153rd day

Serum		Transplant urine	
		C inulin (ml./min./1.73 M²)	8.9
		C PAH (ml./min./1.73 M²)	36
BUN (mg. %)	34	C urea (ml./min.)	8.7
pH	7.4	Filtration fraction	27
CO_2 (mM/L.)	20	Average volume (ml./min.)	2.3
Na (mEq./L.)	135	Na conc. (mEq./L.)	5
		K conc. (mEq./L.)	12
		PO_4 (mM/L.)	3.3
		NH_3 (mg. %)	19

It would thus appear that all kidneys were able to respond somewhat to water loading and that the degree of concentration following the administration of Pitressin®, while sub-normal, was slightly greater in the case of the transplanted kidney than the host's kidneys.

6. Urinary sediment

Urine sediment was evaluated at intervals by the semi-quantitative method of Addis (1). Transplant and bladder urine both consistently showed many white and red blood cells, but only the urine from the bladder showed casts. A typical count is that carried out on the 71st day:

TABLE XI

G. W., Case No. 9—The results of an Addis count of bladder and transplant urine on the 71st day

Excretion/24 hrs.	Bladder	Transplant*
Casts	400,000	0
RBC	37,000,000	3,000,000
WBC	2,000,000	2,000,000

* Urinary cultures from the transplant grew out micrococci and *A. aerogenes*.

7. Output of adrenal cortical hormones, and their effect on electrolyte excretion.[15]

Studies were carried out on the excretion of adrenal cortical hormones in transplant and bladder urine, and their effect on the excretion of electrolytes, creatinine, and uric acid. These results may be summarized as follows:

a) After the 51st day the transplant excreted somewhat greater amounts of both 17-ketosteroids and 17-hydroxysteroids than were obtained in the bladder urine.

b) The output of 17-hydroxysteroid was increased markedly in both bladder and transplant urine after the administration of 150 mgm. of hydrocortisone.

c) A similar increase in the output of 17-hydroxysteroids was seen after the administration of 100 mgm. of tetrahydrocortisone.

d) Hydrocortisone, 150 mgm., was infused intravenously over a 12-hour period. A decreased excretion of sodium, and increased secretions of potassium, chloride, creatinine, and uric acid were noted in the urine from both transplant and host kidneys. These changes were more marked in the urine from the transplanted kidney.

8. Complement titre in peripheral blood [16]

No significant change in complement titre was noted in the post-operative period.

9. Hypertension

The patient's hypertension persisted unabated, the transplant having no noticeable effect on it.

10. The appearance of the ureter

The appearance of the tip of the ureter may provide a clue to the condition of the renal circulation, because its entire vascular supply comes from the renal vessels. In Case No. 4, for example, with focal infarction of the kidney the color of the ureter, which had been bright red, became an unhealthy pink. In Case No. 7, with extensive infarction of the kidney, the ureter became black.

In Case No. 9 the ureter continued to look a healthy red, month after month (see Figure 28). Peristaltic waves could be seen involving the tip, and urine was spurted out of the ureter by the force of these waves (see Figure 29).

11. Other considerations

The location of the kidney did not interfere with the patient's ambulation. The polyethylene covering did permit the kidney to slide about some under the flap, however. The patient noticed at an early stage that if he lay well over on his right side the kidney would sometimes slide over and kink the ureter, producing a temporary obstruction. Turning back to the other side would produce a gush of urine. He made every attempt not to sleep on the right side, but the possibility exists that some intermittent obstruction may have occurred when the patient inadvertently rolled over to this side.

On the 153rd day the patient returned to the hospital for a routine check. At that time he was feeling very well, and expressed a desire to get a job. His appetite was excellent, he had gained weight, and his blood urea nitrogen was down to 34 mgm. per cent, the lowest value we had ever obtained on this patient. His hematocrit had risen to 41, and the blood uric acid had decreased to 6.8 mgm. per cent. The transplant was producing 1,500 to 2,000 ml. of urine daily. The ureter appeared of excellent color, as always.

The patient continued to exhibit a severe hypertension of 200–240/120–140. He had been receiving Dibenzyline®, 20 mgm. a day, during most of the time since the transplantation, but it had not been possible to control the hypertension with this drug. On the 100th day after the transplant, rauwolfia serpentina, two tablets daily of

[15] We are indebted to Drs. John C. Laidlaw and Dalton Jenkins for these studies.

[16] Studies carried out by Drs. C. B. Favour and F. Adler.

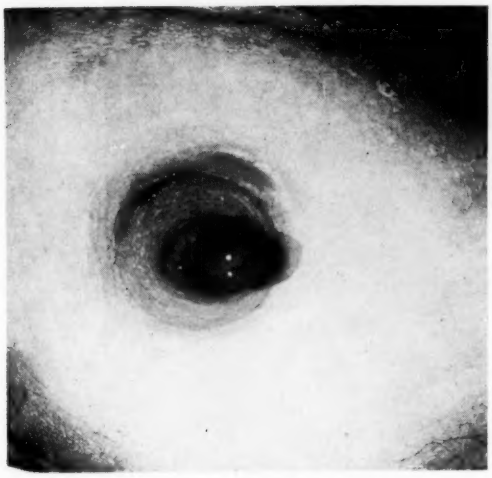


FIG. 28. G. W., CASE NO. 9—VIEW OF URETER ON 91st DAY

Circulation is excellent.

crude extract, was added without noticeable effect. It was decided, therefore, during this hospitalization to increase the dose of rauwolfia. The dose was increased from two to four tablets a day. No significant depression of the blood pressure was noted. On the 7th day of this hospitalization (the 160th post-transplant day) Apresoline® was added in doses up to 150 mgm. a day. On the 9th hospital day the patient noted a slight swelling of the kidney transplant area in the right thigh. By the following day this was definitely noticeable, and the patient had developed a fever of 100.5 degrees. The transplant urine, which had previously contained from 2 to 4 white blood cells per high power field, now contained from 80 to 100. A urine culture showed *Ps. aeruginosa* and *A. aerogenes*. The patient was placed on Chloromycetin®, 2.0 gm. a day.

On the 13th hospital day the patient developed wheezing respiration, and physical examination revealed rales and dullness over both lung bases. On the following day the patient coughed up sputum streaked with bright red blood, and a chest x-ray at this time showed a bilateral hydrothorax with increased vascular markings. It was felt that the findings of swelling of the transplant, hydrothorax, and blood-streaked sputum were compatible with pulmonary emboli originating from the right femoral vein in proximity to the anastomosis of the transplant vein. The patient was started on heparin therapy.

On the 16th day a pleural friction rub was heard over the right posterior chest. The patient continued to run a spiking fever and to have tachycardia and tachypnea.

On the 18th day a pericardial friction rub was noted over the apex. There was pitting edema of the skin overlying the renal homograft, and the output of the transplant had dropped down to around 500 ml. a day. It was decided on this day to explore the right iliac vessels above the graft anastomosis site to see if thrombophlebitis of these vessels was present.[17] At exploration the iliac veins were found to be soft and pliable and there was no evidence of thrombosis or inflammatory process.

On the day following the exploration the urinary output of the transplant, and of the host kidneys as well, dropped off still further. The patient began to vomit coffee ground material and to complain of mid-epigastric pain. Heparin therapy was therefore discontinued. The hematocrit had fallen to 24 by the 22nd day.

The white blood count, which had reached levels of 14,000, came down to 6,000, and the temperature became normal from the 19th day on. The blood urea nitrogen continued to rise, however, and had reached 260 mgm. per cent by the 23rd day. The serum potassium gradually rose. The patient became disoriented and died on 8-6-53, the 23rd day of the last hospitalization, and 5 days short of 6 months from the time of the transplantation.

A complete post-mortem examination was carried out. There was no demonstrable myocardial infarct, but the myocardium was hypertrophied and dilated, and there was a fibrinous pericarditis. There were bilateral pleural effusions, and a fibrinous pleurisy on the right. The lungs were edematous, and there was atelectasis of the posterior portions of the lower lobes bilaterally. No evidence of pulmonary emboli could be found, however. Altered blood was found in the stomach and intestines, but no definite bleeding point was seen.

The patient's own kidneys were markedly contracted, weighing 70 grams each. Microscopically they showed chronic glomerulonephritis of a severe degree.

The renal homograft was extremely interesting. Only the central portion of the kidney was still encased in the bag, the two ends of the plastic sac having become torn

FIG. 29. G. W., CASE NO. 9—LATERAL VIEW OF THE URETEROSTOMY ON THE 105TH DAY

A peristaltic wave is spurting urine out of the end of the ureter.

[17] This operation was carried out by Dr. Joseph Murray.

FIG. 30. G. W., CASE NO. 9—MEDIAL ASPECT OF THE RIGHT THIGH AT POST-MORTEM

An incision has been made through the scar of the original wound. The ureteral stoma can be seen on the anterior surface of the thigh. The polyethylene sac encloses a broad segment of the mid-portion of the kidney. Portions of the upper and lower poles are uncovered by the sac and are in contact with host tissues.

(see figures 30–32). In spite of this, there were very few adhesions over even the uncovered portion of the kidney, perhaps because it moved back and forth under the skin. There was an acute angulation of the ureter 6 cm. from the ureterostomy, with some dilatation above this point. This was consistent with the intermittent obstruction noted by the patient when lying on his right side, and was certainly not the cause either of the renal enlargement or the subsequent oliguria.

The transplant was swollen, pale, and mottled, and the cut surface bulged somewhat—findings felt to be consistent with ischemic nephrosis ("lower nephron

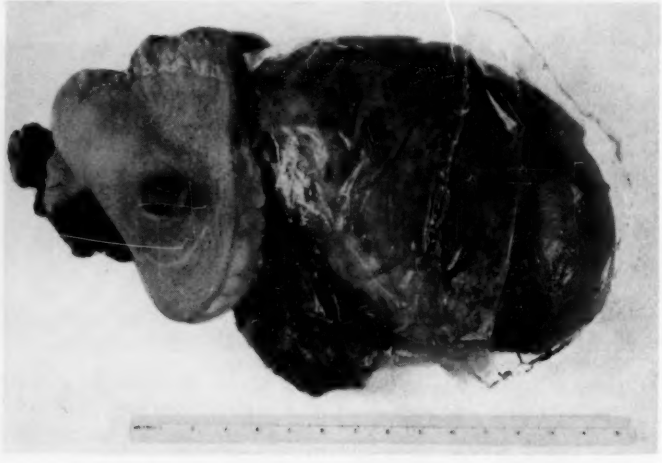


FIG. 31. G. W., CASE NO. 9—THE URETERAL STOMA WITH SURROUNDING SKIN IS SEEN AT THE LEFT.—AT THE RIGHT THE TRANSPLANT MAY BE SEEN, WITH THE POLYETHYLENE SAC COVERING MOST OF IT

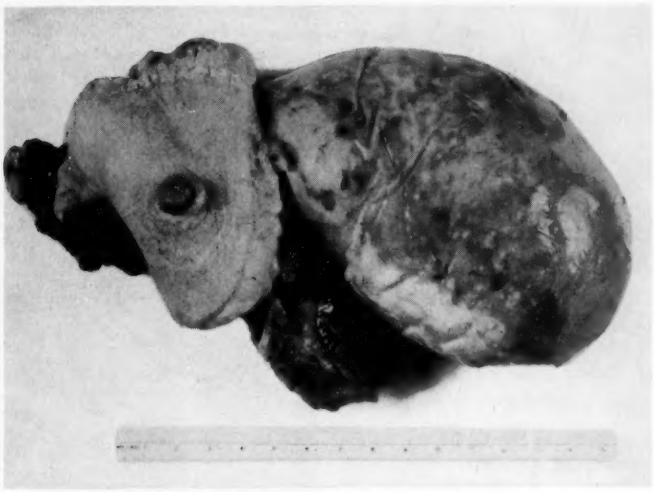


FIG. 32. G. W., CASE NO. 9—THE PLASTIC SAC HAS BEEN REMOVED. THE PORTION OF THE LOWER POLE WHICH HAD BEEN UNCOVERED IS AT THE EXTREME RIGHT. THE MORE MARKED THICKENING OF THE CAPSULE IN THE PART OF THE KIDNEY WHICH HAD BEEN COVERED BY THE SAC IS APPARENT

nephrosis") (see Figure 33). There was no evidence of infection. The blood vessel anastomoses were completely patent, and there was no thrombosis or infarction to be found (see Figures 34 and 35).

The patient's own kidneys showed severe chronic glomerulonephritis.

Microscopically it was noted that the glomeruli in the outer cortex directly subjacent to the polyethylene bag had become fibrosed. There was also fibrosis and marked cellular infiltration of the interstitial tissue in this zone (see Figure 36). These changes were not observed in the deeper layers of the cortex, or in the outer cortex which was uncovered by the bag, and apparently represent a direct reaction to the bag itself. The band of destruction was narrow enough not to constitute a contra-indication to the use of a bag, if such a procedure were shown to have merit. The glomeruli, with the exception of those just mentioned, appeared somewhat ischemic, but otherwise essentially normal (see Figures 37, 42, and 43).

Most of the tubules in the cortex and medulla appeared atrophic in some degree. This was noted most strikingly in the convoluted tubules. Some of the tubules showed casts, and recent degenerative alterations. There was interstitial edema, and focal areas of round cell infiltration

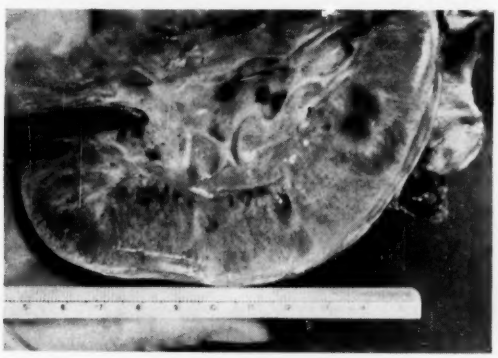


FIG. 33. G. W., CASE NO. 9

The cut surface of the kidney shows pallor and interstitial edema. Accentuation of the architecture and the more marked thickening of the surface tissue where the polyethylene sac was in contact with the kidney can be seen.

FIG. 34. G. W., CASE NO. 9—END-TO-SIDE ANASTOMOSIS BETWEEN THE TRANSPLANT AND HOST VEINS

The dark wavy line represents suture. The endothelium is smooth and glistening throughout, and there is no evidence of thrombosis.

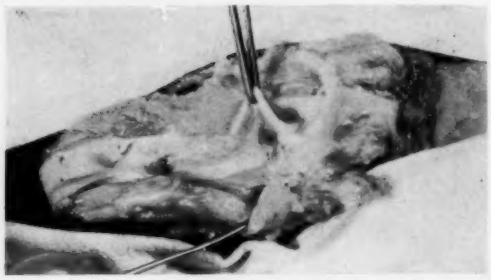


FIG. 35. G. W., CASE NO. 9

The arterial anastomosis can be seen just beneath the tip of the forceps. The endothelium is smooth and glistening throughout. There is no evidence of thrombosis.

(see Figures 37, 41, and 44). The cellular infiltration was sometimes seen around arteries, sometimes around veins, and sometimes just occupying the interstitial space between tubules. There was no consistent relationship between inflammatory cells and any structural unit in the kidney. The tubular degeneration, casts, focal cellular infiltration, and interstitial edema were felt to be consistent with the picture of ischemic nephrosis.

There were striking changes in the intrinsic blood vessels. A severe degree of arteriosclerosis had developed. There was marked thickening of the intima with narrowing of the lumen, in some vessels almost to the point of occlusion. The inner portion of the intima showed dense sclerosis, and the outer portion, just within the inner

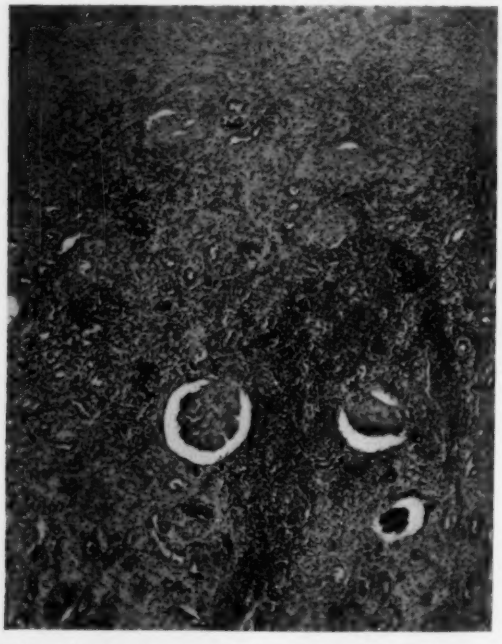


FIG. 36. G. W., CASE NO. 9—THE CAPSULE AND OUTER CORTEX OF THE TRANSPLANTED KIDNEY DIRECTLY SUBJACENT TO THE POLYETHYLENE SAC

There is fibrosis of the glomeruli and interstitial tissue, and cellular infiltration. This picture is in contrast to the lack of glomerular damage in deeper zones of the cortex, or in the outer cortex which was not under the plastic sac. (H & E × 75)

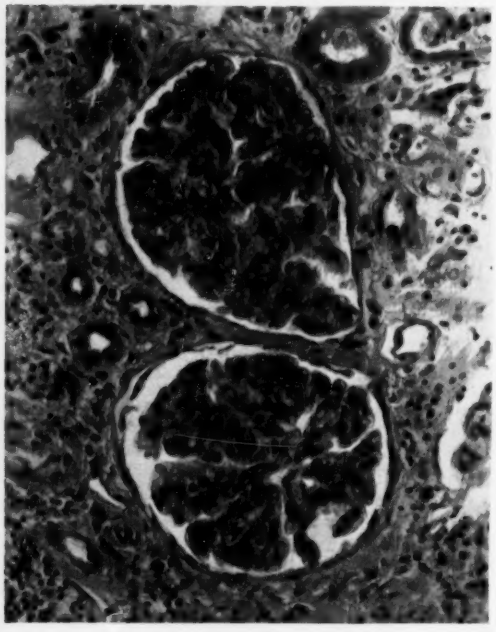


FIG. 37. G. W., CASE NO. 9—MICROSCOPIC VIEW OF DEEPER CORTICAL ZONE

The glomerular tufts appear ischemic, but otherwise fairly normal. There are no proliferative changes present in the glomerular capsule. The epithelium of the tubules is low and the nuclei are variable staining. There is interstitial edema and polymorphonuclear and round cell infiltration. (H & E × 300)

elastic lamina, contained numerous lipid-laden macrophages (see Figures 38, 39, and 40). The media was atrophic.

The inner elastic lamina was fragmented, appeared thicker than normal, and lacked the usual undulating conformation (Figure 39).

Microscopic sections of the ureter showed that the muscle coats of the ureter were still intact. While the patient was living the urine continued to be ejected from the ureter by peristaltic waves (Figure 29), which was evidence for the physiological function of the muscular layers. At the junction of the transplant ureter with the

FIG. 38. G. W., CASE NO. 9—LOW POWER MICROSCOPIC VIEW OF ONE OF THE INTRINSIC VESSELS

Note the marked intimal thickening which has progressed almost to the point of complete occlusion of the vessel. The inner portion of the intima shows dense sclerosis, and the outer portion is filled with lipid-laden macrophages. The media is thinned. (H & E × 75)

skin, the epithelium of the host skin was directly applied to the wall of the ureter without any zone of necrosis between the two. There was some round cell infiltration in this area.

There was no evidence in the transplanted kidney of thrombosis, infarction, calcification, infection, or glomerulonephritis.

DISCUSSION

The cases with measurable function

In eight of the nine cases the homotransplantation was performed in the thigh with anastomosis of the renal vessels with the femoral vessels, and the construction of a skin ureterostomy. Four of these homografts developed measurable function. Despite the small number of cases there are certain similarities between these four which are worth noting. 1) All of the kidneys were small and had vessels whose diameters were equal to or less than those of the femoral vessels to which they were anastomosed. In three instances the small size of the kidney vessels may have been the result of severe mitral stenosis in the donor. In the fourth case the kidney was obtained from a child. 2) All of the patients received testosterone in the immediate post-operative period and for 27 to 62 days thereafter. 3) The blood types of donor and recipient were the same and the bloods cross-matched. 4) All of the kidneys went through a period of anuria from 8½ to 19 days in duration before opening up and beginning to produce urine. One of the kidneys secreted urine for 24 hours before becoming anuric. 5) During the period of maximal performance, each of the four transplanted kidneys developed function which was better than that of the recipient's own.

Beyond these five similarities, there were striking differences between the first three functioning transplants and the fourth one. The first three

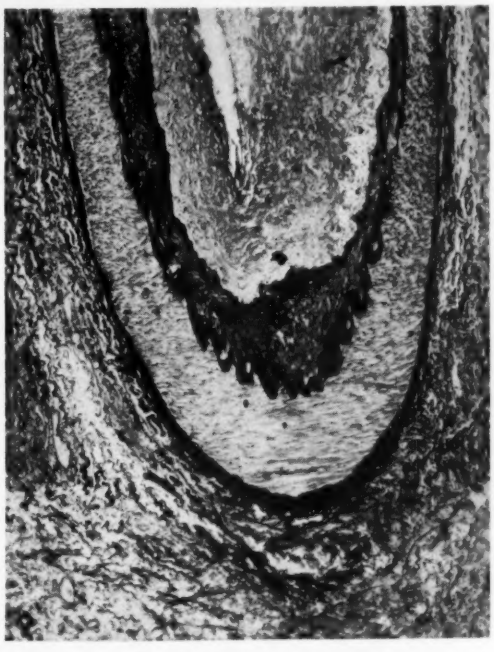


FIG. 39. G. W., CASE NO. 9—A LOW POWER MICROSCOPIC VIEW OF ONE OF THE DEEPER ARTERIES OF THE HOMOGRAFT

Marked thickening of the intima can be seen. The inner elastic lamina is split, and there is atrophy of the media. (Orcein × 75).

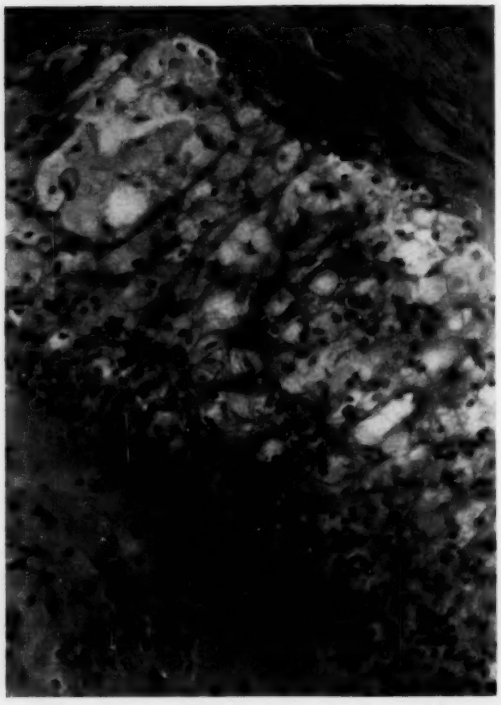


FIG. 40. G. W., CASE NO. 9—HIGH POWER MICROSCOPIC VIEW OF THE WALL OF ONE OF THE INTRINSIC HOMOGRAFT VESSELS

In the lower left hand corner is the inner sclerotic layer of the thickened intima. In the upper right hand corner is the media. Note the marked lipoid infiltration of the inner half of the intima. (H & E × 600)

cases received ACTH and cortisone post-operatively. They all achieved maximum function between the 2nd and 4th week after transplantation, and the urine volume and excretion of metabolites fell off rapidly following this period. The final destructive processes appeared to consist of cortical ischemia, tubular degeneration, and interstitial edema and round cell infiltration. This was followed by scattered thromboses of the small intrinsic renal vessels with associated focal infarcts. All three transplants ultimately became heavily infected. The glomeruli appeared to be relatively uninvolved, except for ischemia, until the destruction of the kidney by infection was nearly complete. The main renal vessels remained patent in one case and had become thrombosed by the terminal stage of the other two cases.

The fourth functioning renal transplant (Case No. 9) was placed in a polyethylene bag at the time of transplantation. No ACTH or cortisone were given. Maximal function was not reached until the 37th day—a time at which the function of the other transplants was already declining—and the function persisted without diminishing for over 5 months from the time of transplantation. The blood urea nitrogen came down progressively from a high of 244 mgm. per cent on the 19th day after the transplantation to a low of 34 mgm. per cent on the 153rd day. Following the failure of the transplant the blood urea nitrogen rapidly rose, over a period of two weeks, to reach a high of 260 mgm. per cent on the day the patient expired.

At necropsy the polyethylene bag was found to be intact over the central portion of the kidney,

FIG. 41. G. W., CASE NO. 9—A LOW POWER MICROSCOPIC VIEW OF THE CORTEX OF THE TRANSPLANTED KIDNEY

An area of focal round cell infiltration can be seen occupying, in this instance, a perivascular position. There is atrophy of the convoluted tubules, and interstitial edema. (H & E × 75)

FIG. 42. G. W., CASE NO. 9—A HIGHER MAGNIFICATION DETAIL OF FIGURE 41

The glomerulus appears essentially normal. There is atrophy of the convoluted tubules, and lymphocytic infiltration. (H & E × 266)

but to be torn over both poles. The microscopic findings in this kidney were somewhat different from the other three cases. The tubules showed signs of acute ischemic nephrosis, and there was interstitial edema and focal cell infiltration as in the other cases. There was, however, no significant infection, no thrombosis, no infarct, and no necrosis. The blood vessels had developed a severe degree of intimal thickening. There was some glomerular damage in the outer layers of the cortex in that portion of the kidney which was covered by the bag, but none in the deeper layers of the cortex, nor anywhere in the parts of the kidney which were not covered by the bag. The bag itself was presumed to have caused the glomerular changes.

Cases without measurable function

Five of the nine cases did not develop *measurable* function. In the first of these (Case No. 1) it was impossible to assess function adequately because the transplant was placed in the renal fossa, and the pelvis of the kidney was anastomosed to the ureter of the host. Extravasation of the urine and infection occurred. The transplant did not excrete dye or maintain the patient. Microscopically, the transplanted kidney showed an ischemic nephrosis with early reparative changes, after 37 days in the host. There was relatively little interstitial infiltration. There was no thrombosis or infarction.

In two other instances the homograft was obtained from a donor whose blood type was different from that of the host. In the first case (Case No. 2) a total infarction of the kidney occurred at sometime between the 3rd and 13th day. Marked local edema and hemorrhage in the wound undoubtedly played some part in the failure of this graft. It was decided, therefore, once again (Case No. 7) to attempt transplantation of a kidney from an incompatible donor. This kidney excreted a few milliliters of clear urine during the first 4 days of the "anuric period." The urine became bloody by the fifth day and dropped off to

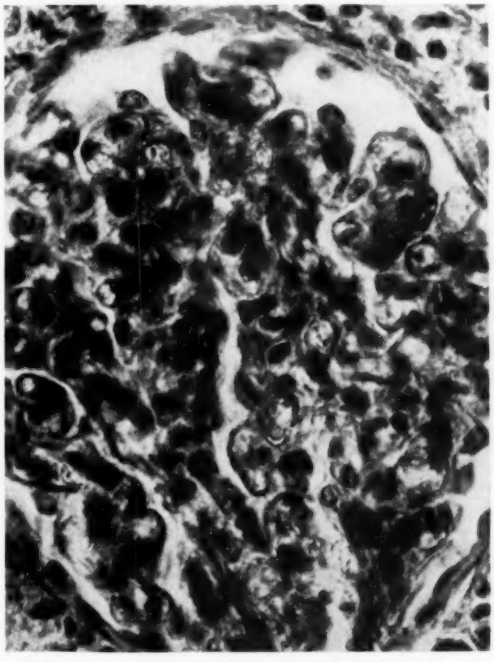


FIG. 43. G. W., CASE NO. 9—A HIGH POWER VIEW OF THE GLOMERULUS IN FIGURE 42, SHOWING NO ESSENTIAL CHANGES FROM THE NORMAL (H & E × 750)

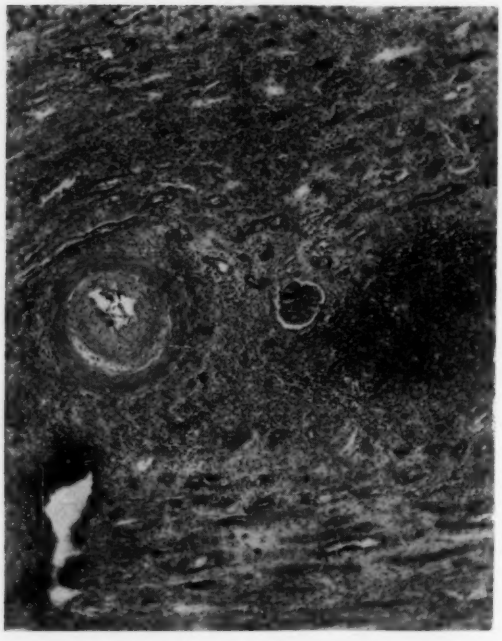


FIG. 44. G. W., CASE NO. 9—A LOW POWER MICROSCOPIC VIEW OF THE CORTEX OF THE TRANSPLANTED KIDNEY

Here a focal area of round cell infiltration surrounds a vein. An adjacent artery shows no periarterial round cell accumulation. Tubular atrophy and interstitial edema can be seen. (H & E × 75)

nothing shortly after that. Total infarction of the kidney ultimately occurred, necessitating removal on the 17th day. Unfortunately this was not a "pure" experiment and we cannot be sure that we can attribute the failure to the blood incompatibility. The following variables may also have played a part: 1) The donor was a hypertensive patient, as was the donor in Case 2. In neither of these two cases did the immediate circulation of the transplant seem to be quite as good as in the others; 2) The kidney was perfused, as was the kidney in Case 2. This was done because of the blood incompatibility. None of the other kidneys was perfused; 3) There was an anomalous vessel going to the lower pole of this kidney and when the circulation had been re-established, the portion of the kidney supplied by this vessel remained dark and in obvious danger of infarction; 4) No cortisone, ACTH, or testosterone was given to this patient, in contrast to most of the others.

In Case No. 6, local edema, hemorrhage, and pressure seemed to have contributed to failure of the graft, which was finally destroyed by infection and infarction.

In Case No. 8, the recipient's uremia was on the basis of periarteritis nodosa. She had been completely anuric (and maintained by dialysis of the artificial kidney) for 45 days at the time of transplantation. A biopsy of her left kidney prior to the transplantation showed severe renal damage, with inflammatory changes in the walls of the blood vessels, hypercellularity and hyalinization of the glomeruli.

Following the transplantation the patient remained anuric (maintained by dialysis) and without function from the homograft until her demise 38 days later. At post-mortem a severe glomerulonephritis was seen to have developed in the transplanted kidney, although there was no thrombosis, no infarction, and no significant infection. There was tubular damage, and hemorrhage had occurred into the lumina of many of the tubules. There was very little interstitial round cell infiltration, however, and no periarterial lesions were present. The glomerular damage seemed to dominate the picture here, the other changes appearing to be secondary to the glomerular damage and the anoxia at the time of transplantation. These changes had apparently developed in 38 days, as the other kidney of the donor was examined microscopically and found to be normal, so that the homograft is presumed to have been normal at the time of transplantation. The changes were probably well developed within two weeks or less, because, had they not been, the transplant would have been expected to "open up" after the initial post-operative anuric phase. This picture of the pathology was in marked contrast to that seen in any of the other cases.

The development of glomerulonephritis in a renal homotransplant

Rich and Gregory (107) produced typical periarteritis nodosa in rabbits by injecting horse serum, suggesting that periarteritis is one manifestation of the anaphylactic type of hypersensi-

tivity. Acute, diffuse glomerulonephritis occurred in a number of these animals.

The glomerulonephritis which involved first the recipient's kidneys and later the transplanted kidney, in Case No. 8, appears, likewise, to have had its origin in a hypersensitive state induced by the injections of a foreign protein. In this case some of the clinical manifestations of the disease were ameliorated by the prolonged administration of ACTH. Progressive renal damage occurred, however, while other tissues were being spared—an observation made as well in other cases of periarteritis treated with ACTH. The terminal phases of destruction of the patient's own kidneys appear to have occurred quite rapidly, after a long period of slowly progressive changes—perhaps because the antibody titre suddenly increased. The destruction of the transplanted kidney, in any event, was very rapid.

Simonsen (119–121) describes vascular changes in dog renal homotransplants which he regards as an "experimentally produced periarteritis nodosa." The glomeruli of these kidneys appear normal. No changes resembling periarteritis nodosa were seen in any of our other human transplants.

It is worth pointing out that Case No. 8 was the only one of our cases showing any evidence of primary glomerular involvement. In the experimental work on the pathogenesis of glomerulonephritis reference is made to the role of antigen-antibody reactions as possible etiologic factors in this disease. A brief mention of this work is pertinent to a discussion of the cases reported here.

Anti-kidney antibodies can be produced in one species of animal by the injection of kidney homogenates from another species. A "nephrotoxic glomerulonephritis" is produced when this heterologous kidney antiserum is injected back into an animal of the species originally donating the kidney tissue (79, 88, 122). No such anti-kidney antibodies have been produced by the injection of homologous kidney homogenates alone, although Schwentker and Comploier (112) described their production following the injection of a mixture of homologous kidney tissue and bacterial toxin, and Hedlund (52) achieved similar results with homologous kidney tissue plus hemolytic streptococcus. Cavelti and Cavelti (24–26) have proposed a theory for the origin of human glomerulonephritis which postulates that streptococcal infection may convert kidney tissue into an antigenic form, permitting the development of anti-kidney auto-antibodies. Humphrey (60) was unable to confirm this work Lange, Gold, Weiner, and Simon (71) found auto-antibodies to human kidney tissue in patients with glomerulonephritis, even in the chronic stage, and Lippman, Cameron, and Campbell (78) found a circulating nephrotoxic substance in patients with glomerulonephritis.

It is of note that no changes of glomerulonephritis were seen in the two renal transplants which were performed by us in patients with chronic glomerulonephritis. This was true in spite of the fact that the homotransplant persisted in one of the cases for a period far longer than that which was necessary for the development of the glomerulonephritis in Case No. 8. It is possible that in chronic glomerulonephritis the anti-kidney auto-antibody titre falls to ineffective levels after a time, and that glomerular changes would have been observed had the transplantations been carried out in the acute phase of the disease.

The post-transplant anuric period

All four of the transplanted kidneys which ultimately showed measurable function developed anuria, or marked oliguria, following transplantation, and then "opened up" after 9 to 19 days. This course resembles that seen with acute ischemic nephrosis following anoxic or toxic injury in the normal human. Two of the kidneys began to function immediately after transplantation (Cases No. 5 and No. 6), but stopped secreting after a few hours. One re-opened 13 days later. The other never functioned, probably due to local factors. These two kidneys were both from living donors with normal renal function—one a child and one an adult. Both went through relatively short periods of anoxia (55 and 14 minutes).

It is likely, therefore, that the post-operative anuric phase is due to the prolonged period of ischemia which the transplants suffer. Most of the donors were in profound shock for some period of time prior to their demise, and this may well have contributed to the ischemic nephrosis

which was felt to be the basis of the anuria. This exposure to anoxia was often continued after the completion of the transplantation, because the recipients were severely anemic and suffered from various degrees of shock in the immediate post-operative period. The thought occurred to us that the initial anoxia might in some way delay the destruction of the transplant by immune mechanisms.

The anuric period is not an invariable part of the course of the human renal transplant, however. One of us (JPM) has had the opportunity of observing the result of a renal homograft performed at the Necker Hospital in Paris. The surgical management was undertaken by Drs. Oeconomos, Vaysse, Michon, and Delinotte, and medical care was under the supervision of Drs. Hamburger, Richet, and Antoine. In this instance a sixteen-year-old boy had a right nephrectomy for traumatic hematoma of the kidney, following which absence of the left kidney was discovered. On the eighth day of anuria a normal kidney was removed from the patient's mother and transplanted into the pelvis of the patient by anastomosis with the iliac vessels. The period of anoxia was 53 minutes. A uretero-ureteral anastomosis allowed the patient to void in normal fashion. The mother's blood type was identical with that of the patient. Urine was elaborated from the homograft within two hours after the completion of surgery, and a good urinary volume and function was maintained for three weeks, thereafter, with marked improvement in the clinical condition and azotemia. However, quite suddenly, on the 23rd post-operative day, function of the homograft ceased. There had been no preceding hematuria, infection, or mechanical obstruction. A biopsy at this time showed numerous bloodless vessels and glomeruli, with marked infiltration of the parenchyma by leucocytes, predominantly lymphocytes and plasma cells. There was widespread degeneration of tubular epithelium [18] (101).

Antibody production in nephritis

Patients with Bright's disease show hypoproteinemia and exhaustion of reserve protein (62). This is partly due to decreased protein intake, and partly to urinary protein loss, but apparently also to depressed formation of serum protein in nephritis (13). In the nephrotic syndrome the hypoproteinemia is characterized by very low levels of gamma globulin, in spite of the reversed A/G ratio (85, 86, 125). There is some evidence that under these circumstances antibody formation and resistance to infection is decreased (16, 17, 42, 67, 87, 91), although this has been disputed by others (6, 8, 100). The lack of agreement is dependent partly on the variable degree of hypoproteinemia present in the animals or patients under study, on the type of antigen used to elicit the immune response, and on the means by which the plasma proteins were lowered.

It is possible that a subnormal antibody production may have contributed to the prolonged survival of the renal homografts seen in some of our patients with chronic renal failure, although there is no direct evidence to indicate that this is so. The more rapid destruction of the homograft noted by Michon and his associates (101) in their case may have been related to the fact that the patient was a healthy young boy in acute uremia, with presumably normal immune mechanisms.

The effect of various hormones

ACTH and cortisone were given in four cases, and ACTH alone in two. We can come to no conclusion about the merit of corticoids or ACTH on the basis of these few cases. No ACTH or cortisone were used in Case No. 9, in which the transplant functioned longer than in any of the others. Persky and Jacobs (106) found that ACTH and cortisone in experimental studies did not prolong the survival of renal homotransplants in the dog. Baker, Gordon, Huffer, and Miller (5) found that treatment with nitrogen mustard plus cortisone, with or without splenectomy, significantly prolonged renal homograft survival in the dog. They point out that because all the animals lost weight the results may be due to the poor nutritional status of the treated animals. The effect of ACTH on skin homografts has been debated, but most investigators now feel that it does not significantly prolong the graft take (7, 41). Cortisone has been found by some workers to prolong skin graft survival (9, 10, 15) and by others to have no effect in this regard (41, 133, 134). Because these substances may contribute to the spread of

[18] We are indebted to Dr. Hamburger for furnishing us with details of this case.

infection, and because their contribution to the life of the graft has not been spectacular, it is felt that they do not offer any hope for the prolongation of human renal homografts.

Testosterone was used in all cases, because of the reported finding that this substance helps to maintain renal weight (64, 65), produces renal hypertrophy (113), and protects against tubular damage under some circumstances (53, 55, 113, 114), and for its anticatabolic effect.

Growth hormone was not tried, but studies with this substance might be of interest.

Blood groups

It is generally accepted that blood groups are of no importance in the destruction of skin homografts in the human (48, 96, 138) or tumor or skin grafts in the fowl (49, 66). No studies have been done on the effect of blood groups in renal homotransplantation. Our own results in transplants done in the presence of ABO incompatibility are not conclusive, as previously stated, because other variables were present in the two cases reported here. We have been influenced by these results, however, to the extent that we do not feel that renal transplantation in the presence of blood incompatibility is wise, if only on the basis of possible local damage to the kidney.

Simonsen (121) has been able to demonstrate incomplete immune agglutinins against the donor's erythrocytes in cases of homotransplantation in the dog. He points out, however, that there is no evidence that these antibodies are identical to those destroying the transplants.

It is of interest that all of the patients with functioning renal homotransplants received blood transfusions at various intervals during their postoperative course. No deleterious effect either to the graft or the patient was noted as a consequence of this.

Biological immunity

The acquired immunity theory of homograft rejection was hinted at in the work of Schöne (110), and clarified and strengthened by Medawar (92–98). This concept holds that the homograft is destroyed as a consequence of the development by the host of specific antibodies against it. Evidence for this is to be found in the following observations: 1) Second set homografts from the same donor are destroyed more rapidly than the first set (48, 77, 93); 2) Second set homografts from another donor are also destroyed more rapidly than the first set, but not so rapidly as secondary transplants from the original donor, (95) suggesting that there is some sharing of antigens between individuals; 3) Direct contact between isolated homologous cells *in vivo* does not lead to their destruction (50), suggesting that homograft rejection is a general rather than local phenomenon; 4) Homografts can persist for long periods in areas devoid of lymphatic drainage; in these areas they can still be destroyed by the placing in another site of a second graft from the same donor (97, 102, 118, 124, 137). Under these circumstances, therefore, the initial graft apparently does not elicit an antibody response, but is still vulnerable to antibodies developed by the host against the second graft.

The chief objection to the theory of acquired immunity at the present time is that it has never been possible to detect circulating antibodies either following the initial transplant, or on repeated "immunization" of the animal.

Loeb has devised a theory, on the basis of his work with implants, which states that lymphocytic infiltration, brought about by "differentials," is the direct cause of homograft disintegration. Immune responses are held to be unessential (80–82). We agree with Medawar, Dempster, and Simonsen that Loeb's theory of differentials is very confusing, and at times "impenetrably metaphysical" (121).

Extensive reviews of these and other concepts of the cause of biological incompatibility are available in the literature (31, 39, 80, 81, 94, 121). The acquired immunity theory is outlined here so that our results can be considered in the light of their possible significance to immune mechanisms of homograft destruction.

The enclosure of a renal transplant in a plastic bag.

In Case No. 9 the donor kidney was encased in a plastic envelope at the time of transplantation. This was done in an attempt to isolate it from the local lymphatic drainage, in the hope that this might decrease antibody formation against it. There is a good deal of evidence to show that

lymphatic tissue plays an important role in antibody production (12, 40, 96, 97, 102, 117, 118, 124, 137), and that regional lymphatics are concerned with the development of antibodies against locally injected antigens (37, 38, 90). Since the plastic bag became torn at some time in the postoperative period, we didn't succeed in isolating the kidney permanently. It is possible, however, that the bag may have played some role in delaying the development of the immune reaction. Experiments are under way in the experimental animal to study the effect on the survival of renal homotransplants of their isolation, by various means, from regional lymphatic drainage (59).

A comparison between renal homografts in the dog and in the human.

Recently Dempster (33, 34) and Simonsen (120, 121) have reported on functional, pathological, and immunological features of renal homotransplants in dogs. The results which they obtained that permit a comparison with our cases are summarized in Table XII.

A fever, associated with loss of appetite and apathy has been described by Dempster in dogs (34). Similar findings were usually noted with homograft destruction in the human. When present the fever was always associated with infection. It was apparently of a milder nature than that observed in dogs.

Homograft survival and function in the human was much better than that seen in the dog. The ability of the human kidney to recover from the ischemic nephrosis seen in the immediate postoperative period is evidence both for its adequate viability and for its freedom from overwhelming antibody attack during this period.

The human renal homografts were sometimes densely adherent to the surrounding tissue. In Case No. 4 a thick pseudocapsule had formed. In general the degree of reaction surrounding the transplant correlated with the amount of destruction, infarction, and infection present. In Case No. 9 there were very few adhesions between the transplant and the surrounding tissue, presumably due to the presence of the bag, and the fact that the kidney could move about in the wound. In Case No. 8, too, very few adhesions were present.

The microscopic picture seen in the human renal transplants corresponds in most cases closely to that described in the dog by Dempster (33). It should be noted, however, that the reaction in the human was much less intense and rapid. Thus Dempster (30) described the microscopic pathology of a dog's kidney 5 days after transplantation as follows: "The kidney is enlarged and its architecture grossly altered by edema, hemorrhagic and non-hemorrhagic necrosis. Most of the glomeruli and many tubules appear necrotic. In addition to hemorrhage, the interstitial tissue of the cortex and outer medulla is heavily infiltrated with polymorphonuclear leukocytes, lymphocytes, histiocytes, and plasma cells. Most of the cortical arteries and veins are dilated and are involved in the necrotising process, and a number are thrombosed. No organisms can be seen in a Leishmann stain. The sinus renalis is massively infiltrated with fibrin, red cells, polymorphonuclear leucocytes, and lymphocytes and histiocytes." This can be compared with the picture seen in the human after 37 days (Case No. 1). The glomeruli appeared relatively bloodless, but there was no appreciable inflammation or increased cellularity. The tubules showed a moderate amount of degeneration, which was prominent in the proximal tubules and in the loops of Henle. Calcium oxalate crystals were present in the lumen of the tubules. There was also some regeneration of tubular epithelium which was most noticeable in the distal tubules (see Figure 5). There was very little interstitial infiltration, and no appreciable edema. There was no necrosis or hemorrhage (see Figures 4 and 5). The histologic picture resembled that seen in severe acute ischemic nephrosis (103).

In Case No. 8, also, very little interstitial infiltration was present, and no appreciable edema.

The blood vessel changes were of interest. No changes resembling periarteritis nodosa were seen. This is in contrast to the findings reported by Simonsen (121) in dog homotransplants. These changes were not observed by Dempster in dogs.

What was the cause of the tremendous atherosclerosis seen in the large and medium sized intrarenal vessels in Case No. 9? Both Dempster and Simonsen describe endothelial swelling in the intrarenal vessels in dog homotransplants. Could this have been an extension of a process

TABLE XII

A comparison between experimental renal homografts reported by Dempster and Simonsen and the human renal homografts reported in this paper

Dempster (33, 34)	Simonsen and his associates (120, 121)	Human renal homotransplants reported here
Function and gross observations		
1. *Toxic syndrome:* High fever, loss appetite coincides with disintegration. Infection plays role. Not present if both kidneys out—due hypothermia of uremia.	1. *Toxic syndrome:* High fever associated with degeneration of kidney, confirming Dempster's findings.	1. *Toxic syndrome:* A fever was usually associated with the degeneration of the transplant. Infection was often present, however. The symptoms abated with the removal of the transplant, as reported in dogs by Dempster (46). In one instance marked hypothermia was present (Case No. 8), probably due to uremia.
2. *Survival of kidney* (number of days kidney secreted urine): a) *As 3rd kidney:* 1–5 days (av. 2.2 D). b) *One host kidney out:* 1–8 D (av. 3.7 D). c) Both host kidneys out: 1–10 D (av. 4.4 D).	2. *Survival of kidney:* In most cases urine secretion was not measured accurately. The bilaterally nephrectomized dogs lived 5–12 days, av. 7.8 D. In 7 cases where urine secretion could be estimated lasted 5–12 days, av. 7.8 D.	2. *Survival of kidney:* In the 4 functioning transplants urinary secretion lasted for from 37 to 180 days, with an average of 97 days.
3. *Reactions with surrounding tissues:* Capsule did not adhere to host tissues, except in one case. (Transplants done in neck.)	3. *Reactions with surrounding tissues:* Transplant partly covered by adhesions of omentum. (Transplants done in abdomen.)	3. *Reactions with surrounding tissues:* In cases with the greatest degree of destruction, adhesions to the surrounding tissue were most marked. In Case No. 4 a thick pseudocapsule had formed. Case No. 8 was an exception (see Text).
4. *Period of anuria:* Began secreting almost immediately. Did not again secrete once it became anuric.	4. *Period of anuria:* Began secreting few minutes to one hour. Did not again secrete once it became anuric.	4. *Period of anuria:* 8½ to 19 days, except for one case (Case No. 5) which secreted for 24 hours and then became anuric. The transplants were able to recover from this period of anuria.
5. *Function of homotransplant:* Blood urea rises steadily despite adequate volume from transplant (up to 10 cc./min.) when host is bilaterally nephrectomized.	5. *Function of homotransplant:* Blood urea rose steadily in all 10 cases with bilateral nephrectomy. In one of these there was a slight one day decrease on the 3rd day.	5. *Function of homotransplant:* The blood urea nitrogen came down in two cases with renal homotransplants (Case No. 4 and No. 9). The transplants could be demonstrated to be functioning at about 25% of normal during these periods.
Microscopic pathology		
1. *Primary transplants:*	1. *Primary transplants:*	
a) *Interstitial:* Cellular infiltration almost entirely confined to cortex. Starts as immature plasma cell infiltration around small blood vessels. Later, cells are mature plasma cells and are found everywhere, except medulla. Polys found in terminal stages. Edema not prominent.	a) *Interstitial:* Focal interstitial round cell infiltration, more pronounced cortex than medulla. Localized chiefly around glomeruli and blood vessels. Cells are half lymphocytes and half pyronine-staining mononuclear cells. Terminally, marked edema and infiltration and/or hemorrhage.	a) *Interstitial:* Focal interstitial round cell infiltration, more pronounced in the cortex than medulla. No specific localization. No pyronine stains done. Marked interstitial edema occurred in some cases. Remarkably little interstitial infiltration was seen in 2 cases at the end of almost 40 days.
b) *Glomeruli:* Relatively bloodless. No cellular change either in endothelium or epithelium of tufts. Basement membrane may be somewhat thickened.	b) *Glomeruli:* Normal.	b) *Glomeruli:* Relatively bloodless. No cellular changes, except in Case No. 8 where severe glomerulonephritis developed.
c) *Tubules:* In late stages tubular casts are present, and droplet degeneration present in tubular cells.	c) *Tubules:* In early stages (3–4 days) may have casts, but often normal. Later many casts, sometimes calcified. Tubules may be dilated, or compressed and fragmented by violent interstitial processes. Coagulation necrosis of entire tubule may occur.	c) *Tubules:* Tubular changes in the later stages appear to be the result of ischemic nephrosis. Casts, and some degree of tubular degeneration was always present.
d) *Blood vessels:* Renal artery normal, intrarenal branches, large and small, show endothelial swelling and pyronin-positive cells.	d) *Blood vessels: 3–4 days:* Intima and media of larger vessels normal. Intertubular capillaries show endothelial swelling. *Terminal:* Intima of all sized vessels from capillaries to large arteries shows swelling and pyroninophilia of the endothelium. There is perivascular infiltration and necrosis of the adventitia, resembling periarteritis nodosa. Arterioles and capillaries of glomeruli show no necrosis.	d) *Blood vessels:* No changes resembling periarteritis nodosa were seen. In Case No. 9 marked intimal thickening had occurred in the larger intra-renal vessels, but not in the renal artery. It was less marked or absent in the smaller vessels. This was not seen in the other cases. In the late stages of destruction of most of the other cases thrombosis began in the smaller intra-renal vessels, extending progressively to the larger ones.
e) *Ischemia* causes renal changes.	e) *Ischemia* occurs as terminal event.	e) *Ischemia* may cause renal changes.

beginning, as Dempster suggests, because the small renal blood vessels are antigenic, antibody fixing, and antibody producing? Progressive changes may have occurred in the blood vessel walls in this case over several months, whereas they might not have been seen in the other cases because they didn't survive as long. Against this, however, is the observation that early stages of this lesion were not seen in any of the other cases, and the kidney which survived for the next longest period of time (101 days) did not show any such changes. Were these changes related in

some way to the fact that the patient had chronic glomerulonephritis? This is unlikely, because no glomerular changes of any significance occurred. Could the atherosclerosis have been caused by persistent severe hypertension applied to a kidney from a host with chronic cardiac failure and hypotension? This we must regard as the most likely cause.

If the development of acquired immunity is the underlying cause of homograft failure, what is the immediate cause of the renal transplant destruction? Simonsen (121) believes that the host's antibodies act in the interstitial tissue to produce allergic vascular lesions resembling periarteritis nodosa. This produces interstitial edema and hemorrhage, interfering with the nutrition and function of the tubules. Direct tubular damage by the antibody cannot be ruled out. Dempster (33) considers that the antibody acts on the small blood vessels of the cortex to produce a generalized vascular spasm, most marked in the tuft capillaries. The swelling and differentiation of the endothelial cells contributes to the slowing down of the renal circulation. The decreased blood flow through the tuft leads to decreased urinary excretion and generalized tubular damage.

The histological picture seen in the human homotransplant seems most in line with Dempster's theory. The changes seen in the uninfected transplant are those of severe ischemic nephrosis, and are compatible with a markedly decreased glomerular blood flow. Certainly in Case No. 9 the severe atherosclerosis must have contributed to the decreased blood flow.

Summary of the observations made herein which relate to immune mechanisms of homograft destruction.

1) The rapid development of glomerulonephritis in a kidney transplanted to a patient with periarteritis, and the absence of this finding in transplants done in patients with chronic glomerulonephritis suggest that the titre of anti-glomerular antibodies in this latter disease must be at ineffective levels.

2) The absence of changes of periarteritis in transplants done in patients not suffering from this disease is in contrast with the reported presence of these changes in dog homotransplants (121).

3) The question is raised whether the period of anoxia suffered by the transplants prior to the re-establishment of a blood supply, or the post-transplantation ischemic nephrosis are able, in any degree, temporarily to depress the antigenicity of the kidney, or the ability of the host to form antibodies against it? No answer is available to this question at the moment.

4) The question is also raised whether there is a decreased ability of the patient with chronic nephritis to form antibodies against homotransplants, which permits a prolonged survival of transplants in these patients?

5) There is evidence that skin shares antigens with leukocytes, but not with erythrocytes (96). No deleterious effect upon the transplant was noted in those patients receiving multiple blood transfusions, nor were there any transfusion reactions. If antigens are shared between different individuals, those which are present in the blood do not seem to have any significance in homograft rejection.

6) Cortisone and ACTH appeared to have no effect on the survival of human renal homotransplants. All of the long term survivals received testosterone, but no conclusion can be reached as to its possible effect.

7) In general, the histologic changes seen in the transplanted human kidney are similar to those reported by Dempster in the dog. The ability of the human kidney to recover from a prolonged period of post-transplantation anuria is unique, however, as were the appearance of glomerulonephritis in one case and arteriosclerosis in another.

8) The immune response to a transplanted kidney in the human is less intense than that in experimental animals reported in the literature, and those in our own experience (59). We cannot agree, on the basis of the foregoing study, with Loeb, who states that the higher in the phylogenetic scale an animal is, the more highly developed is its ability to form antibodies; nor with Dempster, who on accepting this dictum (33) states that "sufficient experiments have been carried out to make it quite clear that any attempt at homotransplanting kidneys in humans, at this stage, is doomed to failure and would be a dangerous procedure" (30).

What is the status of human renal homotransplants at the present time?

Simonsen (119–121) believes that the only real hope of making homotransplantation of the kidney a feasible procedure in cases of chronic uremia in the human has to be based on the following conditions: "First, that the most important individual-specific antigens are shared by the kidney and RBC, so that a gross biological incompatibility can be eliminated by detailed blood group determination; secondly, that the kidney's defense reaction against the recipient's antibodies and/or antigens can check a less significant biological incompatibility without thereby destroying the kidney, and thus provide a more or less complete adaptation to the recipient."

From a practical point of view this offers little hope. In the first place enough human homotransplantations of skin have been done to indicate that there must be a large number of individual specific antigens in the general population not to have encountered compatible pairs by random selection. In the second place we found it extremely difficult to obtain kidneys from donors with the same ABO and Rh groups as the intended recipient. Several of our proposed recipients died in the hospital while awaiting a donor of the right blood type. Therefore, even if one were able to identify a donor whose blood (and renal) antigens were almost identical to those of the recipient, it would be unlikely that the donor kidney would become available at the right time, or that it would be suitable from other points of view (age, infection, cancer, etc.).

Renal homotransplantation has no place in the therapy of human patients at this time. Our own further studies in this field await the outcome of problems now under investigation in the experimental animal. We feel that it will prove valuable, as new data is accumulated in the laboratory, however, to continue to investigate the problem of human renal homotransplantation in the light of these advances. Biological rejection in the human does not seem to be as violent, at least as regards renal transplantation, as that found in the experimental animal.

SUMMARY AND CONCLUSIONS [19]

1. Nine cases of homotransplantation of the kidney in man are reported.

2. Five of the nine transplants did not develop measurable function for various reasons.

3. Four of the nine transplants developed measureable function. The kidneys secreted urine for from 37 to 180 days.

4. It is possible for transplants which have been anoxic for 3 to 3½ hours to develop function in the host which is about 25 per cent of normal.

5. All of the transplanted kidneys went through a period of anuria beginning either immediately after transplantation or 24 hours later. The anuria lasted from 8½ to 19 days in the four cases which ultimately showed measurable function. This was felt to be due to the ischemic interval incident to and following transplantation. The ability of the kidney to recover from this anuria has no parallel in animal experiments.

6. The general pathologic picture which accompanies homograft failure in the human is qualitatively similar to that seen in experimental animals, but appears much more slowly. Pathological changes were observed in two human renal homotransplants, however, that have not been seen in animal experiments:

a) Severe acute glomerulonephritis developed within 38 days in a homograft performed in a patient with polyarteritis nodosa.

b) Marked atherosclerosis of intra-renal vessels occurred within 183 days in a homotransplant taken from a patient with mitral and aortic stenosis and placed in a patient with severe hypertension.

7. Renal homograft rejection in the human is much less violent and slower to develop than it is in experimental animals.

8. The disease of the recipient appeared to be able to modify adversely—in the case of poly-

[19] Since this paper was written another article has appeared (Murray, G., and Holden, R., Am. J. Surg., 1954, **87**, 508.) dealing with renal homotransplantation in the human. Transplants were performed in four cases, with death of the patient in three. In the fourth case the transplanted kidney is thought to be viable and effective after fifteen months. No function studies or biopsies are available, however, to indicate conclusively that the kidney is alive and functioning.

arteritis nodosa—the course of the transplanted kidney. The question is raised as to whether there is a depressed antibody formation in chronic renal disease which can favorably influence transplant survival by diminishing the host reaction against the transplant.

9. Although auto-antibodies against renal tissue are said to be present in the blood of patients with chronic glomerulonephritis, they did not lead to the development of glomerulonephritis in kidneys homografted to patients with this disease.

10. An attempt to reduce the immune response against a renal homograft is described.

11. ACTH and cortisone did not appear to exert any pronounced beneficial effect on the survival of human renal transplants.

12. At the present state of our knowledge, renal homotransplants do not appear to be justified in the treatment of human disease.

13. It is our conviction that this experience serves to indicate certain important species differences in renal homograft response, and that as cellular and animal experimentation progresses, constant cross-check with clinical experience is vitally essential to progress in this field of work.

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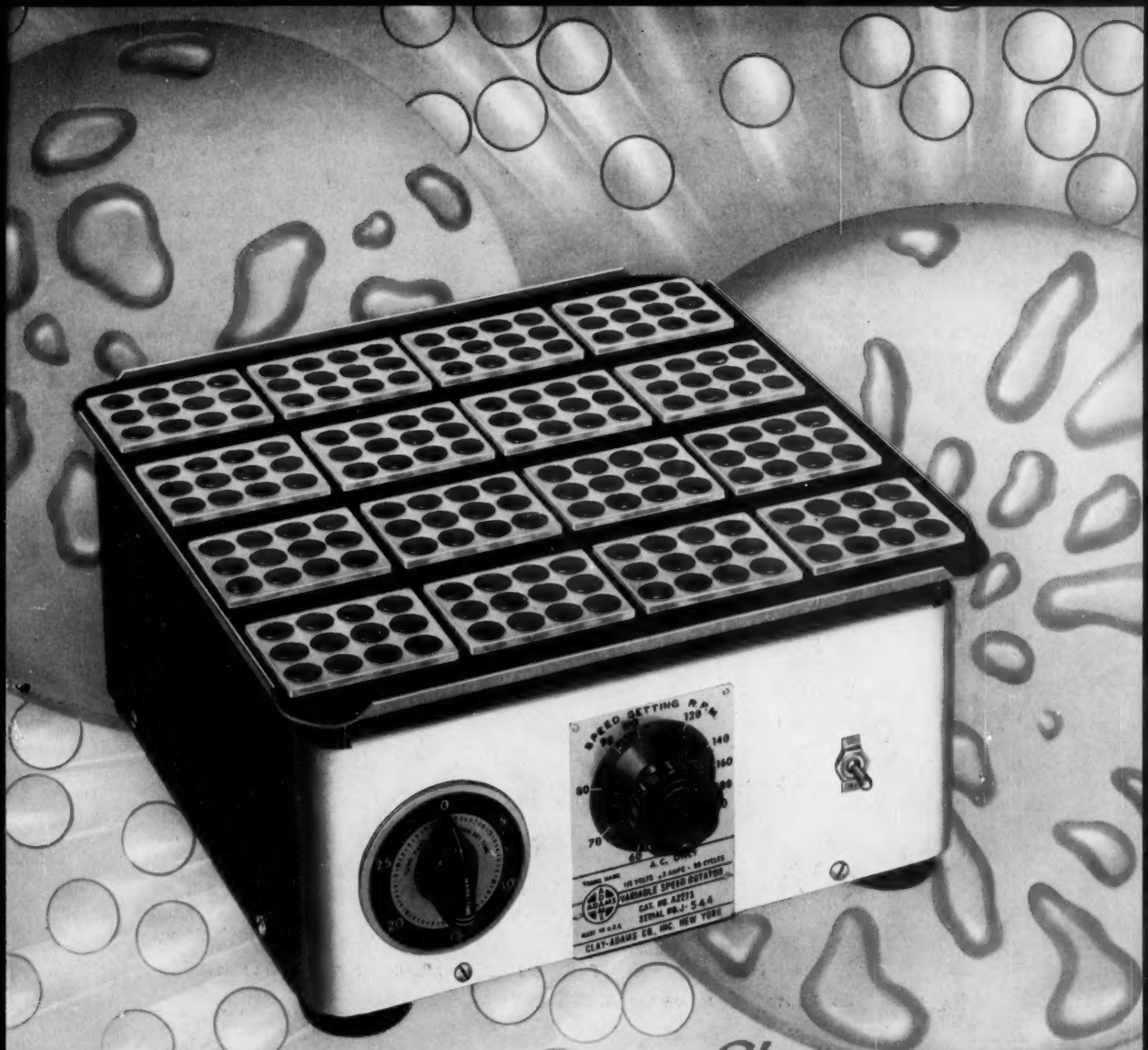

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